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OF THE

AMINO ACIDS AND PROTEINS

With ADDENDUM, pages 1033-1290 Inclusive of Some of the Advances Since 1937

Edited by

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OF THE COLLEGE OF PHARMACY



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CHARLES C THOMAS

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INTRODUCTION TO SECOND EDITION

It is the fate of every book which deals with subjects that are as active as that of the amino acids and proteins, that it does not represent the status of the subject on the day of publication. The past three years have witnessed rapid and, at times, revolutionary progress. To attempt to revise the Chemistry of the Amino Acids and Proteins in toto would be an enormous task and probably unwise at the present time. At the suggestion of the publisher, Mr. Charles C Thomas, the present addendum has been prepared in order to acquaint the student and worker of today with some of the advances that have been made since 1937 when the original manuscript was submitted to the publisher. No attempt has been made in the addendum to cover the literature completely. For the student and worker who desire more complete information, the current journals that publish articles in this field, the Annual Review of Biochemistry, and Chemical Abstracts must be consulted.

Despite rapid advances in certain aspects of amino acids and proteins, other phases of the subject, as was pointed out in the preface to the original edition, still leave much to be desired. Many of the proteins have not yet been prepared in a state of purity that meets the most rigorous specifications of the chemist. There has been a noticeable lag in the perfection of methods for hydrolyzing proteins that do not lead to loss or destruction of amino acids. A good deal of progress has been made in devising techniques for the analysis of amino acids; however, much remains to be done. It appears probable that all of the amino acids occurring in proteins have not yet been discovered. Finally, the structures of proteins still remain to be established by synthesis. Until information on these subjects becomes available, the goal eventually desired still lies far afield.

Due to the amount of new material, some chapters of the addendum are necessarily much longer than others. A few of the chapters needed little or no extension. The numbering of the chapters is the same as in the Chemistry of the Amino Acids and Proteins.

To all of the contributors who have so generously given of their time, we express our thanks. We also desire to thank the publisher, Mr. Charles C Thomas, for suggesting and making possible the publication of the addendum.

INTRODUCTION

If the publication of the Chemistry of the Amino Acids and Proteins has in any way stimulated interest and has assisted anyone in obtaining a better understanding of the subject, we feel that our efforts have been worth while.

CARL L. A. SCHMIDT

May 30, 1942

PREFACE

During the past twenty years information regarding the properties and the behavior of the amino acids and proteins has not only expanded considerably, but has also increased in exactitude. Extensive investigations have been directed to the study of the physico-chemical and thermodynamic properties of amino acids and proteins. This information has done much towards promoting a better understanding of these substances in life processes.

The hypothesis of the zwitterion structure of amino acids is now so generally accepted that it is no longer regarded as a theory. Better methods for isolating and synthesizing amino acids and peptides have been devised. Nutritional studies have not only shown the indispensability of certain amino acids, but have also led to the discovery of hitherto unrecognized ones. Important progress relative to the metabolism of the amino acids in the animal organism has been made. The cooperative efforts of biochemists and immunologists have brought out the importance of the chemical constitution of proteins to immunological specificity.

As is the case with other scientific subjects, the desired information as to the behavior of the amino acids and proteins is by no means complete. Large and important gaps need to be filled. A perfect method for hydrolyzing proteins that does not lead to destruction of or changes in the component amino acids has still to be devised. Very few analyses of the amino acid content account for all of the nitrogen present in proteins. This is probably due in part to the inaccuracies of the available analytical methods. Although better methods for synthesizing many of the amino acids have been described, the methods whereby racemic amino acids may be resolved into the optically active components have not been improved so as to give approximately quantitative yields.

The stereochemical structures of the proteins are unknown. With the discovery of the presence of carbohydrate-containing groups in certain of the proteins, the problem has become more complex. However, some progress in this direction has been made with the development of the carbobenzoxy method for synthesizing peptides and the application of the X-ray method to the analysis of chemical structure. Information regarding the mode of combination

of the metallic elements with proteins, both from a qualitative and quantitative standpoint, needs to be expanded. Thermodynamic and physico-chemical constants for some of the amino acids and for many of the proteins are still to be determined. Many of the studies relating to the physico-chemical behavior of amino acids and proteins have been carried out on dilute solutions. Experimental work in which the concentrations of these substances simulate those found under natural conditions needs to be carried out. Nutritional studies on amino acids have, in large part, been limited to the requirements of the rat. The needs of other species of animals still await investigation. The precise chemical constitution of biologically important substances such as the toxins and antitoxins, certain of the hormones, and the enzymes, all of which are probably protein in nature, needs to be determined. An adequate knowledge of many of the problems in biology awaits a more thorough understanding of the behavior of the amino acids and proteins.

The literature appertaining to the various aspects of amino acids and proteins is so scattered that it has appeared worth while to collect the information and to present it to the reader in an integrated form. Whenever possible, fundamental and important data have been collected and presented in graphical or tabular form. The importance of data that have been obtained by sound procedures cannot be overemphasized. The presentation of available data will incidentally serve as a guide to the gaps which are still to be filled.

A discussion of the chemistry of the enzymes might properly have been included within the scope of this book. This would have necessitated expanding an already large volume. The subject has therefore been presented only so far as it may relate to the structure of certain peptides.

For purposes of convenience, most of the organic formulas given in the text have been presented in the orthodox manner. It is recognized, however, that in the future these formulas may have to be written in conformity with the views expressed by Lowry and Sugden and by Sidgwick.

The present treatise represents a cooperative effort on the part of a group of individuals whose scientific interests lie, in large part, in the field of the amino acids and proteins.

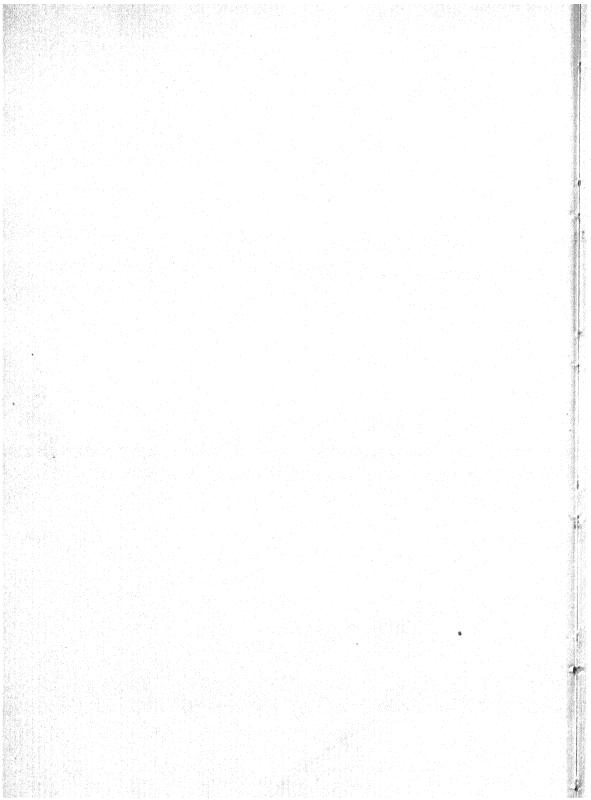
Our thanks are due to those who have so generously given of their time and efforts in contributing to this volume. Without their cooperation our task would not have come to fruition. We are in-

PREFACE

debted to the Editors of the Journal of Biological Chemistry for permission to use copyrighted material from the journal. Free use has been made of material from journals which are not copyrighted. Our thanks are given to the authors of articles in the various journals from which data have been taken. We shall be grateful to those who will direct our attention to any errors or omissions which may occur in the pages of this volume. Dr. Frank W. Allen has given unsparingly of his time in reading and checking the manuscripts. Ruth Goodman has contributed much time and effort to the preparation of manuscripts from this laboratory, and to preparing the index. Dr. D. M. Greenberg gave a great deal of help in various ways during the early part of our work. Others too numerous to mention have, in one way or another, been of help. Mr. C. C Thomas, the publisher, has extended all facilities at his command. The George Banta Publishing Co. has done a painstaking job. To all we express our thanks.

CARL L. A. SCHIMDT

Berkeley, California March 7, 1938



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THE CHEMISTRY $\qquad \qquad \text{OF THE} \\ \text{AMINO ACIDS AND PROTEINS}$

With ADDENDUM
Inclusive of Some of the Advances Since 1937

I submit a body of facts which cannot be invalidated. My opinions may be doubted, denied, or approved, according as they conflict or agree with the opinions of each individual who may read them; but their worth will be best determined by the foundation on which they rest—the incontrovertible facts.

WILLIAM BEAUMONT, M.D.:
"EXPERIMENTS AND OBSERVATIONS ON THE GASTRIC
JUICE AND THE PHYSIOLOGY OF DIGESTION,"
Plattsburgh, 1833.

PART I. CHEMICAL STATICS OF AMINO ACIDS AND PROTEINS

CHAPTER I

HISTORICAL

BY CARL L. A. SCHMIDT

(Division of Biochemistry, University of California Medical School, Berkeley)

1. INTRODUCTION

The wide distribution of proteins as constituents of both animal and plant cells as well as their indispensable relationship to the requirements of animal life has made them the unconscious as well as the conscious objectives of chemical investigation through the ages. The manufacture of cheese and the preparation of glue, the tanning of skins, the discovery that ammonia could be obtained by the distillation of horn or of dung, the use of egg white, blood, and gelatin for the clarification of turbid solutions, and the coagulation of protein foods by heat are applications of protein chemistry. These processes are the result of shrewd chance observations made by individuals whose names are, unfortunately, not recorded in the annals of chemical history.

The story of the proteins and amino acids is one of slow but steady progress. Some of the discoveries were the result of chance observations, others came about as the result of the patient unravelling of clues which indicated the presence in certain proteins of a hitherto undescribed compound and its absence in others. Other discoveries were dependent on the introduction of a new method of attack. It is possible that all of the amino acids which occur in nature have not, as yet, been discovered. The subject is still in active flux. Future progress will, to a large extent, depend upon a knowledge of the methods which were employed in the past as well as in the discovery of new tools. A retrospect of a subject constitutes a proper introduction to a view toward future progress.

2. THE DISCOVERY OF THE AMINO ACIDS

Modern protein chemistry dates from the year 1820 when Braconnot isolated glycine from gelatin by hydrolysis with sulfuric acid. The discovery was one of chance. The hydrolysis was carried out in the attempt to find out whether gelatin would, like wood,

bark, straw, and hemp, yield sugar on acid hydrolysis. The crystals which Braconnot obtained, after neutralizing the sulfuric acid with lime and permitting the concentrated fluid to stand, possessed a sweet taste and he therefore named it "sucre de gelatin" which the Germans translated into "leimzucker." He did not determine that glycine contained nitrogen. Its composition was not known until 1838 when Mulder carried out an analysis. Two amino acids were discovered prior to this date. Their discovery did not come about, however, by a purposeful splitting of the protein molecule. Cystine was isolated from a urinary calculus by Wollaston in 1810, and nine years later, while studying the types of fermentation concerned in cheese making, Proust obtained leucine. Wollaston did not recognize the relationship of cystine to the protein molecule nor did he determine that it contained sulfur. Prout's analysis in 1820, while quite exact, did not show the presence of sulfur. This fact was announced in 1837 by Baudrimont and Malaguti. Although the presence of cystine as a constituent of proteins was suspected by many workers, its actual isolation from proteins did not come about until 1899 when Mörner obtained it by hydrolysis of horn. Three causes contributed to the failure: (a) loss of cystine in the bulky calcium sulfate precipitate formed on neutralization of the sulfuric acid which was used as hydrolyzing agent, (b) reduction of cystine to cysteine with consequent increase in solubility. This was due to the use of tin along with hydrochloric acid in the attempt to prevent humin formation. (c) failure to investigate the proteins of hair, horn, etc., which are rich in cystine. Mörner omitted tin in his hydrolysis. Addition of ammonia in excess prevented precipitation of cystine during neutralization of the acid with calcium carbonate. Subsequent evaporation of ammonia led to deposition of cystine crystals. The presence of cystine in the various stages of isolation was followed by means of the sulfur test. Later, Mörner improved the method of preparing cystine by using sodium hydroxide or ammonia to neutralize the excess of hydrochloric acid. It is of interest to note that about 1899 Embden, then an assistant at Zürich, independently and without knowledge of Mörner's work, isolated cystine from horn.

Proust's discovery of leucine came about as a result of his interest in the processes of fermentation, particularly those to which cheese owes its flavor. Among the products of gluten fermentation he noted several products which, due to their relative insolubility, crystallized out, and which, undoubtedly, were impure leucine.

In 1820, Braconnot isolated leucine by acid hydrolysis of muscle fibre and of wool. He gave the name leucine to designate the white crystals which separated on addition of alcohol to the syrupy concentrate of neutralized protein hydrolysate. In 1827, Braconnot repeated Proust's experiments and obtained a product which he characterized by the name of aposépédine. Both Proust's and Braconnot's preparations were probably crude products. It remained for Mulder, in 1839, to prepare leucine in a pure form, report its correct analysis, and show that leucine and aposépédine were one and the same substance.

The history of aspartic acid began with the discovery of asparagine by Vauquelin and Robiquet in 1806. They obtained this compound by permitting asparagus juice to stand for some time. In 1826. Bacon obtained a crystalline product on addition of alcohol to an aqueous extract of the marshmallow, Althaea officinalis. He considered it to be a salt of malic acid. He gave it the name althèine to designate its origin. A year later, Plisson showed that althèine was the same compound as that which Vauquelin and Robiquet had isolated from asparagus juice. On heating asparagine with lead hydroxide, with subsequent removal of the lead, and concentrating, he obtained a product to which he gave the name of aspartic acid. Its correct empirical composition was established by Liebig in 1838. Ritthausen, in 1868, showed the relationship of aspartic acid to proteins when he obtained it on acid hydrolysis of conglutin and legumin. In isolating aspartic acid, Ritthausen introduced a new experimental method into protein chemistry, viz., the precipitation of the barium or the calcium salt of aspartic acid by means of alcohol. This technique in the hands of the Swedish chemist, Scheele, in 1793, led to the discovery of malic acid. Later, the method was instrumental in the discovery of β -hydroxyglutamic acid by Dakin.

Splitting of casein by alkali fusion led to the discovery of tyrosine by Liebig in 1846. On acidifying the melt with acetic acid and cooling, tyrosine crystals separated. Liebig probably did not at this time regard tyrosine as an amino acid. Two years later, de La Rue isolated tyrosine from an extract of cochineal bugs and showed that it was identical with Liebig's product. He also established its empirical constitution. In 1849, adopting the method of acid hydrolysis used by Braconnot, but employing hydrochloric acid instead of sulfuric acid as suggested by Mulder, Bopp, working in Liebig's laboratory, isolated tyrosine from casein and established

its identity with Liebig's preparation. Like leucine, the discovery of tyrosine was in large part due to its insolubility in aqueous solution at low acidities.

The synthesis of alanine preceded its discovery as a constituent of the protein molecule. It was prepared in 1850 by Strecker according to the well known cyanhydrin reaction by treating aldehyde ammonia with hydrocyanic acid in the presence of excess hydrochloric acid. The discovery was one of chance. The object of Strecker's research was to find a method for synthesizing lactic acid. In 1888, Weyl definitely showed that alanine was a constituent of the protein molecule when he isolated it from the hydrolytic cleavage products of silk fibroin. His method consisted in direct crystallization of the amino acid from the concentrated solution of amino acids after removal of tyrosine. The success of Weyl's work lay in the choice of silk fibroin which is unusually rich in alanine. It is probable that Schützenberger and Bourgeois obtained alanine in 1875. Cramer might have been credited with its discovery in 1865 had he taken the trouble to purify an amino acid fraction which undoubtedly was rich in alanine.

In 1856, Gorup-Besanez isolated valine from an extract of the pancreas. The discovery came about as the result of an extended study of the composition of various glandular extracts. The difficult separation of valine from leucine was accomplished by means of boiling alcohol in which the former is less soluble. Although the relationship of valine to other amino acids which result on hydrolysis of proteins was clearly recognized for some time, the essential step of isolation from a protein hydrolysate became possible only after the development of the method of fractional distillation of the esters of the amino acids by Emil Fischer. This method led to the discovery by Fischer of three amino acids in protein hydrolysates, viz., valine and proline in 1901, and hydroxyproline in 1902.

The amino acid, serine, occurs only in small amounts in most proteins. It was a stroke of good fortune, therefore, that Cramer, in 1865, had chosen to investigate silk gelatin which contains about six per cent of serine. His method of isolating serine from the hydrolytic cleavage products of sericin was to remove tyrosine by crystallization. Further crystallization of the mother liquor yielded crystals of serine. A period of thirty years elapsed before serine was again reported in protein hydrolysates. This time Fischer and Skita isolated it from silk by the method of fractional distillation of its ester.

Fractional crystallization of a protein hydrolysate likewise led to the discovery of glutamic acid by Ritthausen in 1866. He was fortunate in working with gliadin, since this protein is exceptionally rich in glutamic acid. The discovery was not, however, entirely one of chance. Ritthausen observed that his protein hydrolysate contained an acid sufficiently strong to decompose calcium carbonate. Concentration of the protein hydrolysate yielded tyrosine, together with more soluble substances. Fractional recrystallization yielded glutamic acid. With the introduction of the method of hydrolyzing proteins with hydrochloric acid in the presence of tin by Hlasiwetz and Habermann, in 1873, a convenient method of separating glutamic acid as the hydrochloride became available.

The discovery of phenylalanine in the etiolated sprouts of Lupinus luteus was announced in 1879 by Schulze and Barbieri. This is another instance in which splitting of the protein molecule was accomplished by proteolytic enzymes occurring in the tissues from which the amino acid was isolated. It is altogether probable that a number of amino acids which have been described as occurring in plant or tissue extracts, but which have not as yet been isolated from proteins by acid or alkaline hydrolysis, owe their existence in the free state in tissue extracts to hydrolytic cleavage by naturally associated enzymes. The method used by Schulze and Barbieri was one of fractional crystallization in which it was necessary to remove leucine, tyrosine, and aspartic acid in order to purify phenylalanine. Two years later, Schulze and Barbieri obtained phenylalanine by hydrolysis of the protein of squash seed.

Continuing his work on the composition of extracts of etiolated seedlings, Schulze, together with his pupil, Steiger, announced in 1886 the isolation of the amino acid arginine from etiolated lupine seeds. The discovery was the result of the introduction into protein chemistry of phosphotungstic acid as a reagent. Schulze had previously known that phosphotungstic acid would precipitate nitrogenous substances from plant extracts. The precipitate, however, contained various nitrogenous substances, among them, peptones. In isolating arginine, Schulze and Steiger first treated the plant extract successively with tannic acid and lead acetate which removed the more complex nitrogenous products including the peptones. Addition of phosphotungstic acid gave a precipitate that, on decomposition with calcium hydroxide, yielded arginine. Nine years after the discovery of arginine by Schulze and Steiger,

Hedin isolated arginine from among the hydrolytic cleavage products of protein.

In order to understand his discovery, it is necessary to review the events which led to the discovery of lysine by Drechsel in 1889. Three factors contributed to this. The first was the realization that amino acids other than those which had thus far been isolated must be present in proteins, since only a small fraction of the total protein nitrogen could be accounted for in terms of the known constituent amino acids. The second factor was the use of phosphotungstic acid which Schulze had used as a precipitating agent for the isolation of arginine. The third was the demonstration that proteins could be hydrolyzed with hydrochloric acid without loss of carbon dioxide which was found to take place in alkaline hydrolysis. Drechsel hydrolyzed casein, added phosphotungstic acid, and decomposed the precipitate with barium hydroxide. The excess of this reagent was removed and the solution was acidified with hydrochloric acid. On concentrating, a crystalline substance separated. This was finally purified as the platinum salt and was shown to have the composition now ascribed to lysine. Besides lysine, Drechsel obtained a second substance that, from its elementary analysis, he considered to be a homologue of creatine and creatinine. He named this substance lysatinine. His conclusions seemingly received added support when, on hydrolysis with barium hydroxide, urea was obtained. Lysatinine, as we shall see, was impure arginine. The problem was not, however, solved at once. In the course of further observations as to the distribution and nature of lysatinine, Siegfried, a pupil of Drechsel, made the important observation that the phosphotungstic precipitable base formed an insoluble amorphous silver salt. Siegfried did not follow up this clue. It was later destined, in the hands of Kossel, to serve as the key to the method of separating the basic amino acids. Thus the opportunity of adding arginine to their discoveries was lost to the Leipzig School. Meanwhile, in 1891, Schulze and Likiernik demonstrated that arginine like lysatinine yielded urea on alkaline hydrolysis.

A young man who had been associated with Drechsel was S. G. Hedin. He had prepared lysine from the tryptic digest of fibrin, but failed to obtain lysatinine. On returning to Lund, he demonstrated that lysatinine silver nitrate, which he obtained from the hydrolysate of horn, contained lysine. He repeated the experiment on a larger scale with the difference that no alcohol was added to facili-

tate crystallization. The crystalline product that slowly separated proved to be identical with Schulze's arginine. Further studies by Hedin showed that Drechsel's lysatinine was a mixture of arginine and of lysine and that Drechsel's failure to obtain arginine in a pure form was due to the following facts: the acid silver nitrate salt of lysine and the silver nitrate salt of arginine separated when silver nitrate was added to a solution of the bases, with subsequent addition of alcohol and ether. These substances are the more insoluble forms of the double salts of these amino acids. In 1897, Schulze and Winterstein demonstrated that, besides urea, arginine yields ornithine on alkaline hydrolysis.

The third member of the hexone bases, histidine, was discovered almost simultaneously but independently in 1896 by Kossel and by Hedin. Kossel had been interested for some time in the basic proteins of fish sperm to which Miescher had given the name protamin. Kossel hydrolyzed sturin, the protamin of the sturgeon, with sulfuric acid and, after addition of barium hydroxide in excess and removal of barium sulfate, added mercuric chloride. On decomposition of the precipitate, histidine hydrochloride was obtained. Meanwhile, Hedin had accumulated a considerable quantity of an amorphous precipitate which had formed when silver nitrate was added to a solution of the free bases derived from proteins. This precipitate was decomposed with hydrogen sulfide and a sulfur-containing compound was removed by addition of lead acetate. After removal of lead and subsequent addition of silver nitrate, ammonia was carefully added which resulted in the formation of a voluminous precipitate. On decomposing the precipitate with hydrochloric acid and concentrating, crystals of histidine hydrochloride were obtained. It is of interest to note that the method employed by Hedin for the isolation of histidine is still used for the isolation of this amino acid. It is of further interest to note that during his work with Drechsel on lysine and lysatinine, Siegfried had obtained a product whose analysis differed but little from that of histidine hydrochloride. He failed, however, to follow up the clue and thereby missed the opportunity of being the discoverer of this amino acid.

The presence of iodine in certain tissues was the clue which led to the discovery of diiodotyrosine and of thyroxine. In 1894, Drechsel hydrolyzed the axial skeleton of a small coral *Gorgonia cavolinii* with hydrochloric acid. On prolonged heating, iodine was evolved. This suggested gradual decomposition of an unstable com-

pound. Drechsel next showed that the iodine was in organic combination and the organic compound could be precipitated with silver nitrate. The presence of lysine, tyrosine, and leucine indicated that the substance composing the horny skeleton was protein in nature. The isolation of diiodotyrosine was effected by precipitating it from the alkaline hydrolysate with an excess of silver nitrate. The precipitate was subsequently treated with cold nitric acid and, after removal of silver sulfide and silver iodide, ammonia was added. On decomposing the precipitate with hydrochloric acid and concentrating, crystals were obtained. Recrystallization from water gave a pure product. Drechsel announced his discovery of the new amino acid in 1896.

During the winter of 1895–96, Baumann announced the discovery of iodine in the thyroid gland. The discovery was one of chance. It came about when Baumann fused thyroidin, a product obtained from the thyroid gland, with sodium hydroxide and potassium nitrate, dissolved the melt in water, acidified with nitric acid, and, on shaking the solution with chloroform, obtained a violet color. This discovery gave further impetus to the attempt to ascertain the active principle of the thyroid gland. Drechsel's diiodotyrosine, as well as a number of synthetic compounds containing iodine, did not, on testing, bring forth the physiological responses characteristic of thyroid tissue. The isolation of the iodine-containing principle, which characterizes the physiological effect of thyroid tissue on metabolism, was effected in 1915 by Kendall. He had at his disposal unusual facilities for carrying on his investigation on a large scale. To minimize decomposition of the iodine-containing compound, he employed alkaline hydrolysis. Two factors aided in the isolation. The first was the ability to follow the product through the various fractions of the hydrolysate by means of iodine determinations, and the second was its relative insolubility in acid and alkaline solutions. On the basis of the pine splinter test, Kendall concluded that thyroxine contained an indole ring. He decided that thyroxine was trihydro-triiodo-oxyindole propionic acid. As a result of a brilliant series of investigations, Harrington in 1926 demonstrated the incorrectness of Kendall's formula and established the structure of thyroxine as being β -[3:5-diiodo-4-(3':5'-diiodo-4'-hydroxy-phenoxy)-phenyl]- α -amino propionic acid.

Proline is the second amino acid that was obtained synthetically before its presence as a product of hydrolysis was recognized.

It was first synthesized in 1900 by Willstätter who, at that time, was interested in the constitution of hygric acid, a substance obtained by oxidizing hygrine and cuscohygrine, alkaloids which occur in Peruvian cusco leaves. A year later, Fischer obtained it from a casein hydrolysate by fractional distillation of the esters. In 1902, Fischer announced the isolation of hydroxyproline from a gelatin hydrolysate by the method of ester fractionation.

The discovery of tryptophane was the successful outcome of a search for a substance whose presence in the protein molecule had been determined by means of color reactions. One of the first of these was a series of color changes which varied from rose red to violet on addition of chlorine to autolysates of various organs. Another was the Adamkiewicz test that Hopkins and Cole showed. in 1901, was due to the presence of glyoxylic acid in the acetic acid employed as reagent. Another clue was the production of indole when protein digests were subjected to putrefactive processes. An indication that the chromogenic substance might be an amino acid was forecast by the observation of Hopkins and Cole that the glyoxylic acid test persisted after the disappearance of the biuret test in the course of protein hydrolysis. The test was especially pronounced when hydrolysis was effected by trypsin. The success of Hopkins and Cole's search for the chromogenic substance lay in the use of mercuric sulfate as a precipitating reagent. The isolation was accomplished by addition of mercuric sulfate to a tryptic digest of casein acidified with sulfuric acid, subsequent removal of tyrosine and cystine, further addition of mercuric sulfate, and subsequent decomposition with hydrogen sulfide. On evaporation of the solution, which was carried out with frequent additions of alcohol to prevent decomposition, crystals of tryptophane were obtained. The discovery was announced by Hopkins and Cole in 1901.

The story of the discovery of isoleucine is one of accurate polariscopic observation. Fischer had noted that the optical rotation of the leucine fractions of the esterified amino acids varied with each subfraction. Attempts to separate the substance responsible for this from leucine failed. In 1903 F. Ehrlich investigated the nitrogenous residue of beet sugar molasses. By the use of alcohol containing a little ammonia he was able to crystallize a product that, in its elementary composition, was identical with that of leucine. Its optical rotation, melting point, and solubility in water differed somewhat from those of leucine. On preparing the copper

salt of these crystals, Ehrlich noted that a part was fairly soluble and the other part insoluble in water. It was evident that he was dealing with an isomer of leucine. The key to the problem came when Ehrlich found that the copper salt of the isomeric leucine was soluble in methyl alcohol. In order to make certain that isoleucine was a split product of proteins, Ehrlich prepared isoleucine from a pancreatic digest of fibrin and showed its identity with the newly discovered amino acid.

The foundation for the discovery of a dicarboxylic amino acid in the protein molecule, other than aspartic and glutamic acid, was in a sense laid as early as 1873 when Hlasiwetz and Habermann suggested that glutamic and aspartic acids were probably combined in the protein molecule as acid amides. In attempting to correlate quantitatively the dicarboxylic amino acid content of proteins with the amount of ammonia that was set free on hydrolysis, Osborne, Leavenworth, and Brautlecht noted a number of discrepancies and announced, in 1908, that these proteins probably contained another as yet unrecognized dibasic amino acid. They did not follow up the clue. In 1914, apparently without knowledge of Ritthausen's discovery that the calcium salts of the dicarboxylic amino acids are insoluble in alcohol, Foreman applied a similar method to a casein hydrolysate and obtained indirect evidence of the presence of a hitherto unaccounted for amino acid. He was unable to carry the investigation to completion. Dakin's discovery of β-hydroxyglutamic acid in 1918 came about as the result of the introduction of his method of fractionating protein hydrolysates by means of butyl alcohol, in which solvent the dicarboxylic amino acids are insoluble. Glutamic acid was removed by direct crystallization as the hydrochloride, the remaining dicarboxylic amino acids were precipitated as the calcium salts from alcoholic solution, and aspartic acid was removed from this mixture as the lead salt. After removal of a small amount of a basic substance by means of phosphotungstic acid, β-hydroxyglutamic acid was isolated as the silver salt. Dakin also established its constitution.

The discovery of methionine by a man who was primarily interested in bacteriology came about as a result of an investigation in which it was noted that certain protein hydrolysates stimulated the growth of hemolytic streptococcus while others failed to do so. It was found that casein hydrolysates supplied the necessary factor. Mueller determined that mercuric sulfate precipitated the active substance. However, all of the known amino acids which were pre-

cipitated by this reagent were ineffective as growth promoters. A clue to the nature of the amino acid was obtained when Mueller found that one of his crude fractions contained sulfur in a form other than that of cystine. Further work involved repeated use of the salts of mercury as reagents. The yield was about 0.2–0.4 per cent of the casein. It probably does not represent the total methionine content of casein. It is a curious fact that, although the discovery of methionine was the outcome of a search for a growth promoting principle which occurs in casein, methionine, when obtained in purified form, had no particular influence on the growth of streptococcus. At the present time it is recognized that methionine is one of the indispensable amino acids for the mammalian organism.

The discovery by W. C. Rose and his coworkers, in 1935, of β -hydroxy- α -amino-n-butyric acid (threonine) in casein is another illustration of how nutritional investigations have led to the discovery of an amino acid. He found that the butyl alcohol fraction of hydrolyzed casein contains a substance which exerts a remarkably stimulating effect upon the growth of rats. Later this fraction was shown to contain two amino acids, one of which was identified as isoleucine. The new amino acid was obtained by removal of the less soluble amino acids by crystallization, preparation of the copper salts of the remaining materials, and rejection of those which are relatively insoluble in water. After removal of copper from the water soluble salts, the aqueous solution was extracted with butyl alcohol. After removal of isoleucine, phosphotungstic acid was added, and the filtrate was fractionally crystallized by addition of alcohol. The chemical identification followed.

The discovery of citrulline in water-melon juice and later in the split products of casein by Wada was shortly followed by Krebs' work on the rôle played by this amino acid in the synthesis of urea.

Other amino acids have been described as occurring in natural products. Some of these have not, as yet, been obtained from protein hydrolysates, or, if so, the discovery has not been confirmed. The constitution of others has not been established with certainty. This subject is by no means a closed one.

3. HISTORICAL ASPECTS OF PROTEIN CHEMISTRY

Progress in the field of protein chemistry may be said to have developed in three stages: (a) descriptive, (b) analytical, and (c) physico-chemical. Descriptive protein chemistry originated in

the observations of the middle eighteenth and early nineteenth century chemists whose chief object was to isolate proteins from both animal and vegetable sources. Thus, in 1747, we find an account by Beccari of his experiments in which he isolated gluten from wheat flour. In 1805, Einhof discovered that a part of the wheat gluten was soluble in alcohol, and in 1858 Denis showed that many protein substances of both plant and animal origin were soluble in saline solutions. A year later, Ritthausen began the studies on the vegetable proteins which he extended over a period of many years. He attempted to isolate preparations of the highest purity and his work laid the early foundations for an exact knowledge of the vegetable proteins. However, Ritthausen's work was to some extent discredited when Weyl put forward the idea that, by the use of alkali in extracting proteins, Ritthausen had altered their composition. However, Weyl's real service to protein chemistry was not as a critic but in applying the Denis method of extracting proteins by means of neutral salts to a large number of substances of vegetable origin. The work begun by Ritthausen reached its highest point in the hands of Osborne and his coworkers at the Connecticut Agricultural Experiment Station. Not only did Osborne isolate many new proteins of vegetable origin, but his careful and exact analyses of proteins yielded a precise knowledge of their composition. In collaboration with Mendel, Osborne extended his characterization of the vegetable proteins into the field of animal nutrition, and it is to these research workers that we owe much of our knowledge of the protein requirements of animal life. Later, in collaboration with Wells, Osborne further characterized the proteins by showing a relationship between chemical constitution and immunological behavior.

By way of inventory, it may be stated that when Ritthausen began his work on the vegetable proteins, most of the commonly employed methods of isolating proteins, such as extraction with water, with dilute acid and alkali, alcohol, neutral salt solutions, and the precipitating action of neutral salts, had already been discovered as a result of the work of the descriptive chemists. Protein chemistry at this time included a knowledge of the behavior of the albumins, the globulins, and the prolamins.

Success in the isolation of proteins in pure form was followed by studies of their composition. A new epoch was brought into protein chemistry when Boussingault, in 1836, published elementary analyses of a number of plant proteins. Similar work was carried out by Mulder in 1839 and by Liebig and his pupils in 1841 and in the succeeding years. On the basis of these analyses, Liebig, in 1841, put forward the hypothesis that proteins differed in state and not in substance, or, in other words, that proteins of plant and of animal origin which were similarly characterized were identical. This idea was not wholly original with Liebig. The trend of thought in the work of the early descriptive chemists was to establish points of identity between the animal and the vegetable proteins. This early work laid the rudimentary basis for the classification of the proteins. Liebig recognized four types of protein compounds: plant gelatin, vegetable albumin, legumin or casein, and plant fibrin.

The hypothesis of Liebig was not long lived. With the introduction of a new tool in the form of a method for the more exact determination of nitrogen in organic compounds, the proteins were subjected to a more rigorous analysis with the result that in 1842 Dumas and Cahours were able to show decided differences in the elementary composition of many of the then known proteins. The

foundations of Liebig's idea crumbled.

Modern protein chemistry can be said to have its birth in 1820 with the discovery of glycine as a product of the hydrolysis of gelatin by Braconnot. However, the time was not yet ripe for the weaving of this idea into the larger web of protein structure. To understand the trend of the early thought in the field of protein structure, it is necessary to bear in mind the evolution of the ideas of organic chemistry. In 1832, as a result of their investigation of the oil of bitter almonds, Wöhler and Liebig introduced the idea of the chemical radical. This tool enabled them to characterize a series of chemically related compounds. Thus benzaldehyde was characterized as BzH, benzoic acid as Bz, and benzoyl chloride as BzCl. This idea was at once followed by a search by chemists for similarly related series of compounds in a variety of fields. Mulder's notion was to apply the new idea to proteins in the attempt to characterize them more definitely. On the basis of his analyses of a number of proteins that indicated approximately the same composition, Mulder, in 1839, set up the formula, 2C₈H₁₂N₂+5O, as characterizing the protein molecule. In the following year, as the result of further analyses, he now wrote C₄₀H₆₂N₁₀O₁₂ to designate the organic radical. This radical he called protein from πρωτείοs, meaning primarius or first. According to Webster (dictionary) the word protein is derived from the verb $\pi \rho \omega \tau \epsilon b \epsilon \omega$ rather than from the adjective given above. Fig. 1 contains Mulder's statement.

Mulder believed that this radical was common to all proteins, and further that it was combined with phosphorus and with sulfur. He characterized fibrin and egg albumin by the formula \overline{Pr}_{20} P S₂, and serum albumin by \overline{Pr}_{20} P S₄. Mulder's theory received a setback when Liebig showed that fibrin, when precipitated from a

XXXV.

Veber die Zusammensetzung einiger thierischen Substanzen.

Von

G. J. MULDER.

(Bullet. de Neerlande p. 104.)

Ich habe mich seit einiger Zeit mit der Untersuchung der wesentlichsten Substanzen des Thierreiches, des Faserstoffes, des Eiweissstoffes und der Gallerte beschäftigt. Seit der Bekanntmachung dieser Arbeit fuhr ich fort, diese Körper zu untersuchen. Berzelius theilte mir über die veröffentlichten Resultate einige Bemerkungen mit und ertheilte mir gute Rathschläge, für welche ich ihm meinen aufrichtigen Dank sage.

Das Atomgewicht der Substanz ist nach I. 57971, nach II. 55458, nach III. 53622.

Es ist also kein Zweisel mehr, dass das Atomgewicht gehörig bestimmt ist. Die organische Substanz, welche in allen Bestandtheilen des thierischen Körpers, so wie auch, wie wir bald sehen werden, im Psianzenreiche vorkommt, könnte Protein von $\pi \varrho \omega \tau \epsilon i o c_s$, primarius, genannt werden. Der Faserstoff und Eiweissstoff der Eier haben also die Formel $\overline{Pr} + SP$, der Eiweissstoff der Serums $\overline{Pr} + SP$.

Fig. 1.
(J. prakt. Chem., 16, 129 and 138 (1839).)

hydrochloric acid solution by ammonium carbonate, still contained sulfur. According to Mulder's conception, it should have been only protein oxide. Moreover, Laskowski showed that sulfur-free proteins could not be obtained by simple double decomposition. Mulder's hypothesis finally collapsed when it was not found possible, on treating protein with alkali, to demonstrate free sulfur. In accordance with Mulder's notion, the protein sulfur should have been converted to potassium pentasulfide and, on acidification, free sulfur obtained.

No further clue as to the structure of the protein molecule was

brought to light until 1875 when Schützenberger reported the results of experiments in which he treated various proteins with barium hydroxide and reported having obtained ammonia, carbon dioxide, and oxalic acid. On the basis of these results he postulated that urea groups and oxamide groups existed preformed in the protein molecule. Doubt was cast on Schützenberger's results when they failed to receive confirmation in the hands of Habermann and Ehrenfeld in 1900. However, Schützenberger's guess as to the existence of preformed urea in the protein molecule received confirmation with the discovery of arginine as a constituent of the protein molecule and the establishment of its constitution. The idea of classification was uppermost in Schützenberger's mind. In 1879, he classified the amino acids which he obtained on hydrolysis of egg albumin as belonging to two series of compounds that he termed "leucines" and "leuceines."

A new attack on the structure of the protein molecule developed with the study of the action of the proteolytic enzymes on proteins. An early observation by Meissner, in 1859, showing that proteins were incompletely split by pepsin, was taken up by Kühne in 1876. He was able to show that the product left undigested by pepsin could be split further by being subjected to the action of trypsin with the liberation of certain amino acids. However, the splitting was not complete. Moreover, the third proteolytic enzyme, erepsin, was not known at this time. It was discovered by Cohnheim in 1901. It was natural, therefore, that Kühne and his pupils should attempt to isolate from incomplete protein digests unsplit fragments which could in a sense be considered as units or radicals. However, the great complexity of these fragments, as well as the insurmountable difficulty of separating them in a pure state, made the problem of protein structure appear more complex and did not simplify it.

With the discovery of arginine as a constituent of the protein molecule, a further attack on the structure of the protein molecule was made. This came about as the result of Kossel's study of the protamins. Kossel showed that these proteins were unusually rich in arginine and it was therefore natural for him to conclude that since all proteins contained arginine, the protein molecule was built around a nucleus, the kernel of which might be considered to be arginine. However, Kossel's fame in the field of protein chemistry was not to rest on this hypothesis, since it was soon overshadowed by the concept that the amino acids were linked in the protein

molecule as peptides, but on his discovery of histidine and the accurate characterization of the protamins. In line with Kossel's nuclear concept was the idea proposed by Siegfried that the kyrines, a name that he applied to a protein fraction obtained under certain conditions, constituted the nucleus of the protein molecule. This concept was also destined for an early death.

The root of the peptide concept of protein structure had its origin in the work which Schaal published in 1871. He condensed aspartic acid chloride in a stream of carbon dioxide and obtained a product which later was shown by Grimaux to give the biuret reaction. An equally valuable lead came with the synthesis of hippuric acid from benzovl chloride and silver glycocollate by Curtius in 1881. The clue which led directly to the concept of the peptide structure of proteins was obtained by Curtius two years later when he observed that in aqueous solution ethyl glycocollate tended to form glycocoll anhydride. It occurred to Emil Fischer in 1901 that, if this ring could be opened, a new amino acid that would be one degree more complex than the original would be formed. The ring was broken by boiling with hydrochloric acid. This was the beginning of a long and brilliant series of researches by Fischer and his pupils on the synthesis of a great many peptides. This work placed the peptide concept of protein structure on a certain basis. Perhaps an equally important contribution to protein chemistry was made by Fischer in 1901 in developing the ester method of fractionating protein hydrolysates. The peptide concept was not wholly Fischer's. Hofmeister, in 1902, independently advanced a similar hypothesis. He based his conclusions on the linkage which occurs in leucinimide and on the fact that in the native protein molecule only a small fraction of the total nitrogen occurs as amino nitrogen. In more recent years other concepts, such as the diketopiperazine hypothesis, have been advanced, but the essential features of the peptide concept of protein structure have remained.

The close of the nineteenth century saw the beginning of the application of physico-chemical methods to the study of protein behavior. In 1895, Sjöqvist reported experimental work dealing with the conductivity of egg albumin in dilute hydrochloric acid solutions and, three years later, Bugarszky and Liebermann demonstrated, by means of electromotive force measurements, that egg albumin combines with both acids and bases. In 1899, T. B. Osborne found that various amounts of acids were required to neutralize the basic groups in certain proteins. In the same year,

Hardy published his now classic paper showing that in dilute acidic solutions dialyzed egg white migrates to the cathode under the influence of a direct current, and to the anode when the solution is alkaline. Six years later, he demonstrated that serum globulin behaves similarly. Hardy pointed out that in acidic solution proteins carry a positive charge and in alkaline solution a negative charge. The reaction, when the solution was electrically neutral or when minimum dissociation of the protein occurred, was termed by Hardy the isoelectric point. This concept was put into mathematical language by Michaelis and Davidsohn in 1910.

The trend of subsequent work to characterize the protein molecule with the aid of physico-chemical methods has been undertaken from the quantitative standpoint, and this leads us into the present status of protein investigation. In order to correlate the acid and alkali binding capacity of proteins with their chemical makeup, Robertson, in 1911, put forward the hypothesis that this could be sought for in the peptide groups. On the basis of this theory, proteins should yield complex ions when dissolved in either acid or alkali solution. The thesis of Robertson, while ingeniously conceived, nevertheless has not withstood quantitative scrutiny. It is now recognized that the free carboxyl groups plus the hydroxy group of tyrosine are the seat of the alkali binding capacity, while the free amino groups (ε-amino group of lysine, guanidino group of arginine, and the imino group of histidine) are responsible for the acid combining capacity of proteins.

The investigations of Loeb have done a great deal towards elucidating the behavior of the protein molecule. As early as 1900, in attempting to explain the phenomenon of muscular contraction, Loeb advanced the concept of ion-proteids. Eighteen years later, he began a series of beautifully conceived experiments which emphasized the stoichiometric combination of proteins with acid and with alkali. This work definitely brought the subject into the fold of the crystalloids rather than under the indefinite term of colloids. In 1920, by showing that a measurable potential difference exists across a membrane which separates a protein solution from water, Loeb demonstrated that Donnan's idea of membrane equilibrium, with the aid of which Proctor in 1914 had proposed a theory to account for the swelling of gelatin, could be generally applied to account for the influence of electrolytes on the osmotic pressure of protein solutions.

It is not the purpose of this brief review to include in detail the

work of the many investigators who are engaged in erecting the structure of protein chemistry. More detailed accounts than can be given here will be found in the pages which follow. The story of the chemistry of amino acids and proteins, when completed, will constitute one of the outstanding chapters in the history of chemical science.

4. REFERENCES FOR CHAPTER I

- 1. A more detailed account of the history of the discovery of the amino acids and proof of their structure, including references to the literature, is given by Vickery, H. B., and Schmidt, C. L. A., Chem. Rev., 9, 169 (1931).
- 2. More detailed accounts of the history of the development of special phases of protein chemistry, together with literature reference thereto, will be found in the following references:
 - (1) Mann, G., Chemistry of the Proteids, New York, 1906.
 - (2) Osborne, T. B., The Vegetable Proteins, London and New York, 1912.
 - (3) Robertson, T. B., The Physical Chemistry of the Proteins, New York and London, 1918.
 - (4) Loeb, J., Proteins and the Theory of Colloidal Behavior, second edition, New York and London, 1924.
 - (5) Vickery, H. B. and Osborne, T. B., Physiol. Rev., 8, 393 (1928).

CHAPTER II

THE CONSTITUTION AND SYNTHESIS OF THE AMINO ACIDS

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1. SIGNIFICANCE OF THE TERM, "AMINO ACID"

Strictly defined, an amino acid is any organic acid which has one or more substituent amino groups. Viewed from a different standpoint, an amino acid is a substituted ammonia which contains one or more organic acid groups.

Examples of characteristic types of amino acids conforming to these definitions are: carbamic acid (aminoformic acid, NH₂·COOH), which is unstable in the free state and known only in salt form; glycine (aminoacetic acid, NH₂·CH₂·COOH), which is the simplest naturally occurring amino carboxylic acid; aminomalonic acid (HOOC·CH(NH₂)·COOH), the simplest monoamino dicarboxylic acid; ornithine (α - δ -diamino-n-valeric acid, NH₂CH₂·CH₂·CH₂·CH(NH₂)·COOH), a diamino monocarboxylic acid obtained from proteins; and taurine (β -aminoethylsulfonic acid, NH₂·CH₂·CH₂·CH₂·SO₂OH), an aminosulfonic acid present in combined form in bile.

It is evident that a large number of amino acids may exist because of the numerous possibilities for varying the length of the hydrocarbon chain or the position and number of the amino, the carboxyl, or other organic radicals in the molecule. However, the present discussion is limited to those amino (and imino) carboxylic acids which are constituents of proteins. In this group of compounds are included the generally accepted amino acids and those amino acids whose existence in proteins has been reported but not verified in accordance with the requirements originally outlined by Schulze and Likiernik (1) and re-stated in clarified form by Vickery and Schmidt (2). The postulates set forth by these authors as fundamental criteria on which the acceptance of an amino acid should be based are:isolation of the amino acid from protein hydrol-

ysates by an individual other than the discoverer, establishment of its constitution by synthesis, and proof of its identity with the corresponding synthetic compound.

2 RELATION OF AMINO ACIDS TO PROTEINS

The proteins vary considerably in their content of the twentytwo accepted amino acids. In certain proteins, certain of the amino acids are absent or present only in traces while in other proteins the proportion of particular amino acids is relatively high. The following examples illustrate this varied distribution. Glycine is absent in albumins; it is present in small amounts in the globulins, casein, and the alcohol-soluble vegetable proteins; and it is relatively abundant in gelatin. The alcohol-soluble proteins contain relatively high percentages of proline and glutamic acid while the keratins, the proteins of hair and similar types of epidermal tissue. are characterized by their high cystine content. Gelatin is deficient in cystine and the aromatic amino acids. The protamins, the basic proteins of ripe fish sperm, are especially rich in the basic amino acids, lysine, histidine, and particularly arginine. About 80 per cent of the nitrogen of salmin, the basic protein of salmon sperm, is accounted for by arginine (see Table IV, Chapter IV).

According to recent theories, proteins are composed of amino acid residues which are held in firm chemical union by co-valent bonds. An individual chain of this nature is known as a peptide. The nature of the forces by which chains of peptides are bound together in proteins is not known. However, it is believed that the hydration of carbohydrates and proteins in living tissues is effected by means of the hydrogen bond and it seems probable that peptide chains may be bound similarly by coordination with hydrogen (see also Chapter VII).

As shown by the equations,

$$\begin{array}{ccc} \mathrm{NH_2 \cdot CH_2 \cdot COOH} + \mathrm{NH_2 \cdot CH_2 \cdot COOH} \rightleftarrows \mathrm{H_2O} \\ \mathrm{(Glycine)} & \mathrm{(Glycine)} \\ & + \mathrm{NH_2 \cdot CH_2 \cdot CONH \cdot CH_2 \cdot COOH} \\ & \mathrm{(Glycylglycine)} \end{array} \quad \text{and} \quad$$

$$\begin{array}{c} \mathrm{NH_2 \cdot CH_2 \cdot CONH \cdot CH_2 \cdot COOH + NH_2 \cdot CH_2 \cdot COOH} \rightleftarrows H_2\mathrm{O} \\ \mathrm{(Glycylglycine)} \\ \mathrm{+NH_2 \cdot CH_2 \cdot CONH \cdot CH_2 \cdot CONH \cdot CH_2 \cdot COOH}, \\ \mathrm{(Glycylglycylglycine)} \end{array}$$

¹ The hydrogen bond is discussed by W. A. Rodebush in Chem. Rev., 19, 59 (1936).

the formation of peptides appears to consist in the elimination of water from the amino and carboxyl groups of amino acids to form the peptide linkage,-CONH-. However, it should be remembered that both the laboratory and the biological syntheses and degradations of peptides are more complex than appears from these schematic reactions.

The qualitative and quantitative distribution of amino acids in proteins, methods for the determination of amino acids, and theories of protein structure are presented in Chapters IV and VII.

3. CLASSIFICATION OF THE ACCEPTED AMINO ACIDS

Twenty-one amino acids were accepted by Vickery and Schmidt (2) in 1931 and two years later Schmidt (3) recommended that norleucine be added to this list. The classified list of the twenty-two accepted amino acids is given in Table I. It is evident from the data in Table I that all of the amino acids, except proline and hydroxyproline, contain α -amino groups; that imino nitrogen is

TABLE I

Names and Structural Formulas of the Accepted Amino Acids

1. Alanine or α-aminopropionic acid CH₃·CH(NH₂)·COOH

2. Arginine or α -amino- δ -guanidino-n-valeric acid $\mathrm{NH}_2\cdot\mathrm{C}(:\mathrm{NH})\cdot\mathrm{NH}\cdot\mathrm{CH}_2\cdot\mathrm{CH}_2\cdot\mathrm{CH}_2\cdot\mathrm{CH}(\mathrm{NH}_2)\cdot\mathrm{COOH}$

3. Aspartic acid or aminosuccinic acid HOOC·CH₂·CH(NH₂)·COOH

Cystine or di-(α-amino-β-thiopropionic acid)
 HOOC·CH(NH₂)·CH₂·S·S·CH₂·CH(NH₂)·COOH

 Glutamic acid or α-aminoglutaric acid HOOC·CH₂·CH₂·CH(NH₂)·COOH

6. Glycine or aminoacetic acid NH₂·CH₂·COOH

7. Histidine or α-amino-β-imidazolepropionic acid

8. β -Hydroxyglutamic acid or α -amino- β -hydroxyglutaric acid

 $\operatorname{HOOC} \cdot \operatorname{CH}_2 \cdot \operatorname{CH}(\operatorname{OH}) \cdot \operatorname{CH}(\operatorname{NH}_2) \cdot \operatorname{COOH}$

 Hydroxyproline or γ-hydroxypyrrolidine-α-carboxylic acid HOCH—CH₂

TABLE I—(continued)

10. Iodogorgoic acid or 3:5-diiodotyrosine

$$HO \underbrace{\stackrel{I}{\longleftarrow}} CH_2 \cdot CH(NH_2) \cdot COOH$$

11. Isoleucine or α -amino- β -methyl-n-valeric acid

 $CH_3 \cdot CH_2 \cdot CH(CH_3) \cdot CH(NH_2) \cdot COOH$

 Leucine or α-amino-iso-caproic acid or α-amino-isobutylacetic acid CH₃·CH(CH₂)·CH₂·CH(NH₂)·COOH

13. Lysine or α-amino-ε amino-n-caproic acid

NH2·CH2·CH2·CH2·CH2·CH(NH2)·COOH

14. Methionine or α -amino- γ -methylthiol-n-butyric acid $CH_3 \cdot S \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

15. Norleucine or α-amino-n-caproic acid

 $CH_3 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

16. Phenylalanine or α -amino- β -phenylpropionic acid

$$\bigcirc$$
 CH₂· CH(NH₂)· COOH

17. Proline or pyrrolidine-α-carboxylic acid

18. Serine or α -amino- β -hydroxypropionic acid

19. Thyroxine or β -[3:5-diiodo-4-(3':5'-diiodo-4'-hydroxyphenoxy)phenyl]- α -aminopropionic acid

$$HO \underbrace{\stackrel{I}{\longrightarrow}} O \underbrace{\stackrel{I}{\longrightarrow}} \mathrm{CH_2} \cdot \mathrm{CH(NH_2)} \cdot \mathrm{COOH}$$

20. Tryptophane or α -amino- β -indole propionic acid

$$CH_2 \cdot CH(NH_2) \cdot COOH$$

21. Tyrosine or α -amino- β -(p-hydroxyphenyl) propionic acid

$$HO \bigcirc CH_2 \cdot CH(NH_2) \cdot COOH$$

22. Valine or α-amino-iso valeric acid

 $CH_3 \cdot CH(CH_3) \cdot CH(NH_2) \cdot COOH$

present in arginine, histidine, hydroxyproline, proline, and tryptophane; and that histidine alone contains a tertiary nitrogen atom. The aliphatic amino acids include alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, β -hydroxyglutamic acid, isoleucine, leucine, lysine, methionine, norleucine, serine, and valine, while

Table II

Empirical Formulas, Optical Forms of the Naturally Occurring Substance,
Molecular Weights, and Percentage Composition of
the Accepted Amino Acids

	Empirical formula	Opt. form	Molec- ular weight	Percentage composition			
				C	н	N	О
Alanine	$\mathrm{C_{3}H_{7}O_{2}N}$	d	89.06	40.42	7.92	15.73	35.93
Arginine	$C_6H_{14}O_2N_4$	d	174.14	41.35	8.10	32.18	18.37
Aspartic acid	$C_4H_7O_4N$	d	133.06	36.07	5.30	10.53	48.10
Cystine ¹	$C_6H_{12}O_4N_2S_2$	ı	240.23	29.97	5.03	11.66	26.64
Glutamic acid	$C_5H_9O_4N$	d	147.08	40.80	6.17	9.52	43.51
Glycine	$C_2H_5O_2N$	_	75.05	31.98	6.71	18.67	42.64
Histidine	$C_6H_9O_2N_3$	ı	155.09	46.42	5.85	27.10	20.63
β -Hydroxyglutamic							
acid	$C_5H_9O_2N_3$	d	163.08	36.79	5.56	8.59	49.06
Hydroxyproline	$C_5H_9O_3N$	l	131.08	45.77	6.92	10.69	36.62
Iodogorgoic acid ²	$C_9H_9O_3NI_2$	l	432.91	24.97	2.10	3.24	11.09
Isoleucine	$C_6H_{13}O_2N$	d	131.11	54.92	9.99	10.68	24.41
Leucine	$C_6H_{13}O_2N$	l	131.11	54.92	9.99	10.68	24.41
Lysine	$C_6H_{14}O_2N_2$	d	146.13	49.27	9.66	19.17	21.90
Methionine ³	$C_5H_{11}O_2NS$	l	149.15	40.23	7.43	9.39	21.45
Norleucine	$C_6H_{13}O_2N$	d	131.11	54.92	9.99	10.68	24.41
Phenylalanine	C ₉ H ₁₁ O ₂ N	l	165.09	65.41	6.72	8.49	19.38
Proline	$C_5H_9O_2N$	l	115.08	52.14	7.88	12.17	27.81
Serine	C ₃ H ₇ O ₃ N	l	106.06	34.27	6.72	13.33	45.68
Thyroxine4	C15H11O4NI4	l	776.77	23.17	1.43	1.80	8.24
Tryptophane	$C_{11}H_{12}O_2N_2$	l	204.11	64.67	5.93	13.72	15.68
Tyrosine	C ₉ H ₁₁ O ₃ N	l	181.09	59.64	6.12	7.74	26.50
Valine	$C_5H_{11}O_2N$	d	117.09	51.24	9.47	11.96	27.33

¹ 26.69 per cent sulfur.

histidine, hydroxyproline, iodogorgoic acid, phenylalanine, proline, thyroxine, tryptophane, and tyrosine have either the benzene, phenol, pyrrolidine, indole, or imidazole ring in their molecules. β -Hydroxyglutamic acid, hydroxyproline, iodogorgoic acid, serine, thyroxine, and tyrosine contain hydroxyl groups. Cystine and methionine contain sulfur, while iodogorgoic acid and thyroxine are iodated. Thyroxine is an aromatic ether derivative.

² 58.63 per cent iodine.

³ 21.50 per cent sulfur.

^{4 65.35} per cent iodine.

On inspection of their formulas, it is seen that many of the amino acids are derivatives of propionic acid and hence are structurally related to alanine. By substituting a hydroxyl group for a hydrogen atom of the methyl group, alanine is changed to serine. Similarly, tyrosine is formed by the introduction of a hydroxyphenyl ring, phenylalanine by a phenyl ring, iodogorgoic acid by an iodized phenyl ring, tryptophane by an indole ring, histidine by an imidazole ring, thyroxine by an iodine-containing ring complex, and cystine by sulfur atoms into the molecule of alanine.

The molecular weights, percentage composition, and optical forms of the accepted amino acids are listed in Table II. Only one amino acid, glycine, is optically inactive. β -Hydroxyglutamic acid, hydroxyproline, and isoleucine exist in four optically active forms since each of these amino acids contains two asymmetric carbon atoms. Each of the remaining 18 amino acids has only two optically active modifications owing, in all but one case, to the presence of a single asymmetric carbon atom. Although cystine has two asymmetric carbon atoms, only two optically active isomers are known. A third form, meso cystine, is optically inactive because of internal compensation.

4. CONSTITUTION OF THE ACCEPTED AMINO ACIDS

A. General Principles

In determining the chemical constitution of the crystalline substances which have been discovered among the hydrolytic products of proteins, the first step, and an important one, is the purification of the unknown substance. The elimination of contaminating inorganic salts is readily accomplished by precipitating the amino acid as the insoluble silver, lead, or other heavy metal compound or by recrystallization of the amino acid from water, water-alcohol mixtures, or other solvents. When necessary, the amino acid is converted to an appropriate salt and the latter is purified by recrystallization. Ordinarily, the copper salt, the hydrochloride, or the picrate is used for this purpose although such compounds as the benzoylate, phosphotungstate, flavianate, picrolonate, reineckate, rhodanilate, heavy metal double salt, and other types of derivatives capable of ready reconversion to the original amino acid have been employed. In some instances amino acids are purified by fractional vacuum distillation of their ethyl esters, by selective extraction with partially miscible solvents, or by other special methods of treatment. That some of the purification procedures are inadequate is illustrated by the recent discovery of Mueller (4) that samples of *l*-leucine may contain as much as 9 per cent of methionine.

While melting points have long been used as a criterion of purity for crystalline organic compounds, the so-called melting point values of amino acids are known to vary widely under different experimental conditions. That temperatures reported by different observers are discordant is not surprising since melting, dehydration, decomposition, and sublimation may proceed independently or simultaneously on exposure of amino acids to different temperatures for varying times. These results are not unexpected in view of the profound changes undergone at high temperatures by some amino acids, e.g., glutamic acid and β -hydroxyglutamic acid each loses a molecule of water to form a pyrrolidone carboxylic acid. As has been shown by Dunn and Brophy (5), the melting or decomposition temperatures of the amino acids are much higher than those which usually have been reported, especially when the temperatures at which these changes occur instantaneously, are measured. It has been suggested that the zwitter ion structure of crystalline amino acids is responsible for this effect. On the other hand, concordant values are obtainable by ordinary laboratory methods when the conditions are carefully standardized. While melting temperatures of this character have no fundamental significance, they played an important part in much of the earlier work on the constitution of amino acids. Fortunately, the sharp melting points of amino acid derivatives provided dependable criteria by which conclusions drawn from amino acid melting point data could be confirmed.

The crystalline form of amino acids is sometimes used as an index of purity. However, uncertainties may arise because an amino acid may crystallize from different solvents or from a given solvent under varying conditions of concentration, time, and temperature as needles, plates, or prisms. Furthermore, the characterization of amino acids by this method is sometimes difficult because of the close resemblance between the needle, plate, or prism-like structures of various forms; apparent differences between micro and macro crystals; the dissimilarity of crystals from impure and pure solutions; and decomposition during evaporation of the solvent. Our present knowledge of the crystal habits of the amino acids is in an unsatisfactory state, although Keenan (6) has contributed

photomicrographs of interest in this connection (see Chapter IX). Photomicrographs of a number of crystalline amino acid derivatives have been provided recently by Kirk (7) and co-workers. It is expected that these observations will be of particular importance in identifying arginine and lysine. Although these amino acids were crystallized by Vickery and Leavenworth (8), special experimental methods were required and the free bases could not be maintained readily in the crystalline state.

Recrystallization of amino acids until the solubilities, specific rotations, or specific conductivities are constant, constitutes effective, although tedious, methods for establishing the purity of these substances. More rapid, and what are thought to be more satisfactory, methods for this purpose are (a) titrations in glacial acetic acid with perchloric acid using colorimetric or electrometric methods for the determination of the end point (9), and (b) formol titrations by means of the glass electrode (10).

The second step in establishing the chemical characteristics of an amino acid is the determination of its empirical formula. This is accomplished by (a) macro- or micro-combustion analyses of the purified material for carbon, hydrogen, oxygen, nitrogen, sulfur, and iodine and (b) simple calculations from these data. In the majority of cases, the correct percentages of the chemical elements can be determined without particular difficulty. However, sulfur was overlooked in the earliest investigations of cystine and, for a time, the formula of this amino acid was thought to be $C_9H_{12}N_2O_4S$. The discoverers of thyroxine, Kendall and Osterberg (11), advanced the formula, $C_{11}H_{13}O_3N_2I_3$, but this was later corrected to $C_{15}H_{11}O_4NI_4$ by Harington (12).

The third, and most difficult, step is the determination of the spatial arrangement of the atoms in the amino acid molecule. As a rule, the probable structure of the amino acid was first assigned from a knowledge of the products resulting from reactions of the amino acid with nitrous acid, permanganate, chlorine, hydrogen peroxide, alkalies, reducing substances, molds, or other agents. In order to test the validity of these deductions, the amino acid was synthesized and its chemical, physical, and physiological properties were determined. If the synthetic and the racemized natural product, or the natural form and the comparable isomer, obtained by resolution of the synthetic mixture, were found to be identical, the constitution of the amino acid was considered to be definitely established. That the d or l and dl-amino acids have different

physical properties was not recognized in some of the earlier investigations.

B. Specific Methods

This discussion is limited to some of the more important data from which our knowledge of the constitution of the amino acids has been gained. For more complete surveys of the literature bearing on this topic, the reader is referred to reviews by Plimmer (13) and by Vickery and Schmidt (2).

(1) Alanine. The constitution of alanine was readily determined by comparison of the natural with the synthetic product. The latter was prepared many years before the natural isomer was isolated from proteins. It has been shown that alanine is converted to α -hydroxypropionic acid (lactic acid) by treatment with nitrous acid and that carbon dioxide, ammonia, acetaldehyde, and acetic acid are formed by oxidation with hydrogen peroxide (14).

(2) Arginine. The isolation of urea by Schulze and Likiernik (15) and of urea and ornithine (α - δ -di-amino-n-valeric acid) by Schulze and Winterstein (16) from the alkaline hydrolysate of arginine led to the conclusion that arginine is either α -amino- δ -guanidino or α -guanidino- δ -amino-n-valeric acid. Ornithine, isolated as the dibenzoyl derivative (ornithuric acid), was shown to be identical with the compound which Jaffé (17) obtained from the urine of birds to which benzoic acid had been fed. This hypothesis was supported by later experiments of Schulze and Winterstein (18) who prepared arginine by the evaporation of ornithine and cyanamide (CN·NH₂) and those of Kutscher (19) who obtained guanidine, guanidine butyric acid, and succinic acid by the oxidation of arginine with permanganate. Furthermore, ammonia, ornithine, tetramethylenediamine, and δ -amino-n-valeric acid are known to be formed from arginine by bacterial decomposition.

Definite proof of the position of the guanidino group in the n-valeric acid chain was furnished by Sörensen (20) in 1910. When ornithuric acid is heated with hydrochloric acid, δ -monobenzoylornithine is formed, while treatment with barium hydroxide gives α -monobenzoylornithine. On condensing these isomeric monobenzoylornithines with cyanamide and hydrolyzing the products, only the guanidino derivative prepared from α -monobenzoylornithine is identical with racemized natural arginine.

(3) Aspartic Acid. Clues to the constitution of aspartic acid were obtained by Piria (21) who isolated malic acid from the re-

action products when asparagine was treated with nitrous acid, and by Kolbe (22) who proved that ammonia is not readily formed by the action of alkalies on aspartic acid. The latter observation indicated that the nitrogen of aspartic acid is present in some form other than the amide. Later investigations showed that succinic acid is produced from aspartic acid by bacterial action. The constitution of aspartic acid was proved by Piutti's (23) synthesis from oxaloacetic ester oxime.

- (4) Cystine. The probable constitution of cystine was deduced from the following experimental observations: (a) Formation of lactic acid (CH₃·CHOH·COOH) by the action of nitrous acid; (b) production of ammonia, rather than methylamine, by the action of alkalies; (c) reduction with zinc and hydrochloric acid to a product (later shown to be cysteine, [HS·CH₂·CH(NH₂)·COOH]) which could be re-oxidized to cystine; (d) preparation of β-thioand β-dithiopropionic acids, identical with the synthetic compounds; (e) and oxidation of cysteine to taurine (NH₂·CH₂·CH₂·SO₂OH), presumably through the intermediate cysteic acid [HOSO₂·CH₂·CH(NH₂)·COOH]. A considerable number of these experiments on cystine were performed by Friedmann and coworkers (24). The constitution of cystine was definitely proved by Erlenmeyer's (25) synthesis from benzoylserine ester and phosphorous pentasulfide.
- (5) Glutamic Acid. Evidence for the now accepted structure of glutamic acid was presented by Ritthausen (26), Dittmar (27), and Markownikoff (28). It was shown that glutamic acid is converted to α-hydroxyglutaric acid by the action of nitrous acid and that this hydroxyacid is reduced by hydriodic acid to glutaric acid (HOOC·CH₂·CH₂·CH₂·COOH). In more recent investigations it has been found that succinic, butyric, and formic acids, in addition to other products, are formed from glutamic acid by oxidation with hydrogen peroxide or by the action of bacteria. Final proof of the constitution of glutamic acid was given by Wolff (29) in his synthesis of the compound from levulinic acid.
- (6) Glycine. The structure of glycine was readily determined by its reaction with nitrous acid to form glycollic acid (HOCH₂·COOH) and by comparing the naturally occurring form with the product synthesized from bromoacetic acid and ammonia (30) (31).
- (7) Histidine. The investigations of Herzog (32), Fränkel (33), and particularly those of Pauly (34) and Knoop and Windaus (35) led to the establishment of the correct structure for histidine.

These workers observed that (a) histidine forms a dihydrochloride and a dinaphthalene sulfonyl derivative; (b) histidine is resistant to both oxidation and reduction; (c) histidine reacts with nitrous acid to give β -imidazolelactic acid which may be reduced to β -imidazolepropionic acid; (d) histidine may be oxidized by nitric acid to β -imidazole-glyoxylic, -acetic, and -carbonic acid and (e) histidine is converted by deamination, followed by reduction, to a product which is identical with synthetic β -imidazolepropionic acid. The structure of histidine was definitely confirmed by Pyman's (36) synthesis in 1911.

- (8) β -Hydroxyglutamic Acid. The constitution of β -hydroxyglutamic acid was determined by Dakin (37) who described the properties of this amino acid as follows: (a) strong monobasic acid in aqueous solution, (b) dibasic acid in aqueous formaldehyde, (c) all of the nitrogen is in the α -amino form as shown by the nitrous acid reaction, and (d) one molecule of water is lost and the amino nitrogen is converted to the imino form by exposure to phosphorus pentoxide. It was therefore evident that the dehydrated product was a pyrrolidone carboxylic acid derivative and that the amino group in β -hydroxyglutamic acid is in the γ -position relative to one of the carboxyl groups. It was shown further that β-hydroxyglutamic acid is reduced to glutamic acid, and that it is oxidized to an aldehyde which reacts with p-nitrophenylhydrazine to give the osazone, HOOC · CH₂ · C: (N · NH · C₆H₄NO₂) · CH: (N·NH·C₆H₄NO₂). The formation of an osazone indicates that the hydroxyl group is adjacent to the aldehyde group in the aldehyde acid and to the amino group in β -hydroxyglutamic acid. Since there was no indication of lactone formation, the hydroxyl group was assumed to be in the β - and not the γ -position. Although color tests, presumably indicative of a hydroxyacid, were obtained with resorcinol, β -naphthol, and diazobenzene sulfonic acid, it was shown later by Harington and Randall (38) that one cannot rely upon these color reactions. The structure of β -hydroxyglutamic acid was confirmed in 1919 by Dakin's synthesis from glutamic acid (39).
- (9) Hydroxyproline. It was shown by Fischer (40) in 1902 that hydroxyproline is a hydroxy derivative of pyrrolidine carboxylic acid since it can be reduced to proline. γ -Hydroxyproline was synthesized in 1905 by Leuchs (41) and identified by reduction to proline. By fractional crystallization of their copper salts, Leuchs isolated two products which were assumed to be inactive stereoiso-

meric modifications. In later experiments, Leuchs and co-workers (42–44) concluded that natural hydroxyproline may be either β - or γ -hydroxypyrrolidine carboxylic acid (compounds II and III, page 76), but not the α - or δ -derivative (compounds I and IV, page 76).

The α -derivative was excluded since natural hydroxyproline, when heated with barium hydroxide at 200°, was only partially racemized, indicating the presence of two asymmetric carbon atoms in the molecule. The δ -hydroxy compound was considered improbable because of the known stability of natural hydroxyproline to the action of alkalies. The exact constitution of hydroxyproline has not been determined up to the present time.

- (10) Iodogorgoic Acid. In 1896, Drechsel (45) detected iodine vapors when the skeleton of coral was treated with acid. He isolated a substance of the empirical formula, C₄H₈NIO₂, from the degradation products of this material. Henze (46) found that this substance contained an aromatic group since it gave a positive xanthoproteic test. The synthesis of iodogorgoic acid from tyrosine and iodine was described in 1905 by Wheeler and Jamieson (47). Two years later Henze (48) demonstrated that this substance is identical with the inactive product which Wheeler and Jamieson had prepared by alkaline hydrolysis of coral. Additional proof of the constitution of iodogorgoic acid was presented by Wheeler and Johns (49) who methylated the natural amino acid, subjected it to treatment with hot alkali, and isolated a product which proved to be identical with synthetic 3,5-diiodo-p-methoxycinnamic acid.
- (11) Isoleucine. Ehrlich (50, 51) isolated isoleucine from proteins and the nitrogenous products which are present in beet sugar molasses and proved the constitution of this amino acid. It was found that 1-amino-2-methylbutane $[CH_3 \cdot CH_2 \cdot CH(CH_3) \cdot CH_2 \cdot NH_2]$ and isoleucinimide,

$$\begin{array}{c|c} NH-CO\\ & | & |\\ CH_3\cdot CH_2\cdot CH(CH_3)\cdot CH & CH\cdot CH(CH_3)\cdot CH_2\cdot CH_3,\\ & | & |\\ CO-NH \end{array}$$

shown to be different than leucinimide, are formed by heating d-isoleucine to 200°. The alcohol, d-1-hydroxy-2-methylbutane [CH₃·CH₂·CH(CH₃)·CH₂OH], was obtained from d-isoleucine by the action of yeast. The constitution of the alcohol was determined by oxidizing it to methylethylacetic acid [CH₃·CH₂·(CH₃)CH·COOH].

The structure of isoleucine, which had been deduced from these experimental results, was confirmed by Ehrlich (51) who synthesized it from d-methylethylacetaldehyde, hydrocyanic acid, and ammonia. The mixture of d-isoleucine and d-alloisoleucine resulting from the synthesis, appeared to be identical with that obtained by heating natural d-isoleucine for 20 hrs. with saturated barium hydroxide solution. The d-alloisoleucine, obtained by destroying the natural d-isoleucine in the synthetic mixture by the action of yeast, was shown to be identical with the d-alloisoleucine isolated from the mixture resulting from the racemization of natural d-isoleucine. d-Isoleucine, identical with Ehrlich's natural product, was isolated from a synthetic mixture by Locquin (52), while the complete resolution of the synthetic isoleucine-alloisoleucine mixture and the physical properties of the four optically active isomers (d- and l-isoleucine and d- and l-alloisoleucine) have been described more recently by Abderhalden and Zeisset (53).

- (12) Leucine. Leucine was known to be a constituent of proteins as early as 1820, but more than seventy years elapsed before its constitution was determined. Early workers concluded that leucine was an α -aminocaproic acid since it was oxidized to ammonia and valeric acid, deaminized to a hydroxycaproic acid, and reduced to ammonia and a caproic acid. However, it was not until 1891 that Schulze and Likiernik (1) showed that the racemized natural l-leucine was identical with the synthetic product obtained from isovaleraldehyde, ammonia, and hydrocyanic acid by Limpricht's (54) method; that the products prepared from synthetic and racemized natural leucine by the action of the mold, *Penicillium glaucum*, were identical; and that the same hydroxyacid resulted from the deaminization of synthetic and natural leucine.
- (13) Lysine. The structure of lysine was indicated by its bacterial decomposition to cadaverine (pentamethylenediamine) (55) and its oxidation to glutaric acid. The constitution of lysine was proved by Fischer and Weigert (56) who synthesized it from γ -cyanopropyl malonic ester by treatment with nitrous acid followed by reduction.
- (14) Methionine. The existence of this amino acid in meat infusions and protein hydrolysates was reported in 1921 by Mueller (57). An unknown substance, removable by norit from these materials, was shown to be essential for the growth of hemolytic streptococcus. In later investigations by this author (58), the amino acid was isolated from easein hydrolysates as the mercuric sulfate

complex, purified as the copper salt and the naphthyl isocyanate, and analyzed. The molecule was found to contain sulfur in a highly stable state and nitrogen only in the amino form. It was further demonstrated that this substance is oxidized in the animal body. A compound of the most probable structure, synthesized in 1928 by Barger and Coyne (59), was shown to be identical with the natural product.

(15) Norleucine. Many difficulties were encountered in differentiating norleucine from the isomeric leucine and isoleucine. The presence of norleucine in proteins was first reported by Thudichum (60) who isolated a product of this composition from neuroplastin and found that it had a characteristic solubility. About ten years later, Abderhalden and Weil (61) isolated a similar substance from nerve tissue. The solubility, melting point, specific rotation, and other physical properties of this product differed from those of leucine and isoleucine, but were in close agreement with those of the synthetic product. However, the method by which norleucine (first known as "caprine") was separated from leucine and isoleucine was not described in detail. Subsequently, norleucine was again isolated from nerve substance by Abderhalden and Heyns (62), while Abderhalden and Beckmann (63) sought to differentiate the isomeric leucines by the method used successfully with the valines (64). The fraction thought to contain norleucine was treated with nitrosyl bromide and the resulting α -bromocaproic acid was subjected to the action of ammonia. The rates at which bromine was removed from this compound and the three possible isomeric bromoacids, prepared by synthesis, were compared. However, only small differences in the rates were observed and a definite conclusion could not be drawn from the experiment.

Further evidence that norleucine is a constituent of proteins was reported in 1932 by Czarnetzky and Schmidt (65). d-Norleucine was isolated from beef spinal cord by fractional crystallization of its copper and zinc salts and was purified by ten re-crystallizations of the amino acid from water. The natural d-norleucine and the dl-forms of norleucine, leucine, and isoleucine were exposed to dry ammonia or hydrogen chloride and their dissociation pressures measured. Since the latter were slightly, but definately, different for the three types of chemical substances but identical for dl- and d-norleucine, the presence of norleucine in proteins appears to have been confirmed. In 1933, Schmidt (3) recommended that norleucine be added to the list of accepted amino acids.

- (16) Phenylalanine. The constitution of this amino acid was proved largely by the experiments of Schulze and collaborators (66), Schützenberger (67), and Fischer. It was shown that benzoic acid, benzaldehyde, phenylacetaldehyde, phenylacetic acid, phenylpropionic acid, and phenylethylamine were formed from phenylalanine, as well as from proteins, by oxidation, dry distillation, or bacterial action. The supposition from these data that phenylalanine is α -amino- β -phenylpropionic acid was confirmed in 1882 by Erlenmeyer and Lipp's (68) synthesis from acetaldehyde by the Strecker method and the observation that the synthetic and natural compounds had identical properties.
- (17) **Proline.** The constitution of proline was readily determined by comparison of the natural with the synthetic product which Willstätter (69) prepared prior to the isolation of proline from casein. The probable structure of proline was suggested by the absence of amino nitrogen and the formation of n-valeric and δ -amino-n-valeric acids by the action of bacteria.
- (18) Serine. Cramer (70), the discoverer of serine, found that glyceric acid is formed when this amino acid reacts with nitrous acid. Fischer and Leuchs (71) showed that alanine is produced when serine is heated with hydriodic acid and red phosphorous. The structure suggested by these observations was confirmed by Fischer and Leuch's (71) synthesis from glycolaldehyde by the Strecker method.
- (19) Thyroxine. The conclusions of Kendall and Osterberg (11) that thyroxine is a trihydro-triiodo derivative of tryptophane were shown to be erroneous by Harington (12) and Harington and Barger (72). The latter investigators proved definitely that thyroxine is a diiodomethoxyphenyl derivative of diiodotyrosine. The constitution of this complex amino acid was proved by means of the following observations: (a) The empirical formula was shown to be C₁₅H₁₁O₄NI₄ by combustion analysis; (b) iodine was removed quantitatively by catalytic reduction. The product of this reaction, thyronine (desiodothyroxine), gave a positive Millon's test and was completely deaminized by the action of nitrous acid. These tests indicated that a hydroxyphenyl group and nitrogen, exclusively in the amino form, were present in the thyroxine molecule; (c) fusion with potassium hydroxide at 250° gave p-hydroxybenzoic acid, a small amount of quinol, and the substance, C₁₃H₁₂O₂. Fusion at 310° in hydrogen yielded p-hydroxybenzoic acid and quinol in considerable quantities, toether with ammonia and

oxalic acid. These experiments suggested the presence of two benzene rings, one of which had a phenolic or phenol ether group in the position para to a side chain from which a two-carbon fragment could be split off as oxalic acid; (d) the compound, 4-(4'-hydroxy-phenoxy) toluene (I), was synthesized and shown to be identical

with the substance, $C_{13}H_{12}O_2$, previously prepared by alkaline fusion of desiodothyroxine; (e) the synthetic 4-(4'-methoxyphenoxy) benzoic acid (II) was identical with the acid, $C_{14}H_{12}O_4$, obtained by

$$CH_3 \cdot O$$
 O $COOH$ (II)

oxidation of desiodothyroxine; (f) the synthetic [α -amino- β -4-(4'-hydroxyphenoxy) phenylpropionic acid] (III) was identical with natural desiodothyroxine; (g) the synthetic [4-(4'-methoxy-

$$HO$$
 O $CH_2 \cdot CH(NH_2) \cdot COOH$ (III)

phenoxy) benzaldehyde] (IV) was identical with the aldehyde, $C_{14}H_{12}O_3$, which is formed by the degradation of thyroxine; (h) the

$$CH_3 \cdot O$$
 CHO (IV)

synthetic p-methoxyphenoxycinnamic acid (V) was identical with the unsaturated acid, $C_{16}H_{14}O_4$, obtained during the degradation of thyroxine; (i) on oxidation, compound IV was converted to compound II; (j) from analogy with iodogorgoic acid, the four

iodine atoms in thyroxine were assumed to be in the 3, 5 and 3', 5' positions. The synthetic 3, 5-diiodo-4(4'-methoxy-3', 5'-diiodo-phenoxy) benzoic acid (VI) was identical with the product which was prepared from natural thyroxine by methylation and oxidation of the side chain;

$$CH_{3} \cdot O \underbrace{I}_{I} COOH$$
 (VI)

(k) synthesis of thyroxine by the method shown on page 92; and (l) resolution of the synthetic product and proof that one of the active isomers is identical with the product obtained from the thyroid gland both in its chemical properties and in its ability to accelerate metabolism [Harington (73); Harington and Salter (74)].

(20) Trytophane. As early as 1826, protein digests were known to give characteristic color reactions when treated with bromine water, concentrated acids, and other reagents. However, it was not until 1902 that the crystalline substance (later shown to be tryptophane) which is responsible for these reactions was isolated by Hopkins and Cole (75). Almost thirty years prior to the isolation of tryptophane, the formation of

indole
$$NH$$
, skatole NH

and what were thought to be skatole carboxylic and skatole acetic acids, by the bacterial decomposition of proteins was observed by several investigators. Although Hopkins and Cole assumed from these findings that tryptophane was skatole aminoacetic acid, Ellinger and co-workers (76) concluded from a series of outstanding investigations that the products isolated by former workers were, in all probability, the isomeric indole, rather than the skatole, derivatives. It was only after Ellinger and Flamand (77) synthesized tryptophane, and showed its identity with the natural product, that the correct formula was established.

(21) **Tyrosine.** Although tyrosine was isolated from cheese by Liebig (78) in 1846, it was not until 1882, the date of its synthesis from phenylalanine by Erlenmeyer and Lipp (79), that its constitution was definitely established. Early workers assumed that tyrosine was ethylaminosalicylic acid, but this view was proven to be untenable by the observations of Barth (80, 81), Hüfner (82), Baumann (83), and others. They showed that, on oxidation, tyrosine gave p-hydroxybenzoic acid; on reduction, ammonia rather than ethylamine; and, by bacterial decomposition, hydroxyphenyl-propionic as well as other homologous acids.

(22) Valine. The difficulties experienced by early workers in determining the constitution of valine were caused by the inability to distinguish between the isomeric amino-n-valeric, aminoiso-

valeric, and aminomethylethylacetic acids. Moreover, they failed to recognize that the inactive synthetic product and the active natural form differ in physical properties. The correct structure was finally established in 1906 by Fischer (84) who showed that the racemized natural valine corresponded in properties with synthetic α -aminoisovaleric acid and that d- α -aminoisovaleric acid, obtained by resolution of the synthetic product, had the same specific rotation as valine isolated from proteins.

5. LABORATORY SYNTHESES OF THE ACCEPTED AMINO ACIDS

Laboratory methods for the preparation of the amino acids have been investigated for more than eighty-five years. Early workers realized that the constitution of amino acids isolated from proteins could be adequately verified only by the synthesis of compounds with identical physical properties. Nevertheless, progress was slow at the beginning, and it was forty years after cystine was isolated from a urinary calculus by Wollaston (85), before the first syntheses, that of alanine by Strecker (86) and aspartic acid by Dessaignes (87), were described in 1850. However, synthetic procedures were gradually developed from the investigations of Ellinger, Erlenmeyer, Fischer, Leuchs, Sörensen, Schulze, Strecker, von Braun, Wheeler, and other chemists.

Because of the difficulties inherent in the isolation of some of the amino acids, the inadequacy of many of the older syntheses, and the need for relatively large quantities of amino acids in investigations relating to their metabolic and physico-chemical behavior. the classical syntheses have been re-investigated in more recent times and new methods have been devised. In this work important contributions have been made by Abderhalden, Barger, Harington, Knoop, Marvel, Sasaki, and many other persons. As a result, satisfactory syntheses are available for many of the amino acids although entirely adequate synthetic methods for arginine, cystine, β-hydroxyglutamic acid, hydroxyproline, histidine, thyroxine, tryptophane, and a few other amino acids are still lacking. That relatively little attention has been given in recent years to the synthesis of amino acids may be partly explained by the readiness with which cystine, leucine, glutamic acid, tyrosine and certain other amino acids may be isolated from proteins. However, the fact that syntheses may be expensive, tedious, and productive of racemic mixtures which often require laborious resolution procedures for the preparation of the optically active forms, is also responsible for this condition.

The principal types of chemical reactions, and a number of unique procedures by which amino acids have been synthesized. are discussed later in this section. While there have been surprisingly few changes in the techniques laid down by early workers, a number of important contributions have been made in recent times. Among the latter may be listed (a) the application of mass action principles to syntheses from aldehydes, ammonia, and hydrocyanic acid and from α -halogen acids and ammonia in order that troublesome products formed by side-reactions may be minimized: (b) the substitution of a modified technique for the original Strecker method: (c) replacement of high pressure reactions in closed tubes by reactions in high boiling inert solvents at reflux temperatures and atmospheric pressures; (d) purification of amino acids by fractional crystallization from appropriate solvents in the presence of chloride, bromide, and other ions rather than by removal of the latter as their insoluble lead, silver, and other heavy metal salts: (e) hydrolysis of imides, ethers, hydantoins, azlactones, diketopiperazine condensation products, and other types of intermediate substances with concentrated solutions of the relatively high boiling hydrobromic and hydriodic acids in order to avoid excessive decomposition of the amino acid; and (f) simultaneous, rather than step-wise, reduction and hydrolysis of unsaturated amino acid precursors by the use of hydriodic acid and red phosphorous.

A. General Reactions

(1) Cyanohydrin Synthesis. The aldehyde-ammonia, formed by the reaction of alcoholic-ammonia or an ammonium salt with an aldehyde, is allowed to react with anhydrous hydrocyanic acid or a solution of an alkali metal cyanide. On hydrolysis of the resulting aminocyanohydrin with a strong acid or base, an amino acid with one more carbon atom than the original aldehyde is formed. These reactions (shown below), first used by Strecker (86) for the synthesis of alanine from acetaldehyde, have been employed for the preparation of glutamic acid, glycine, isoleucine, leucine, methionine, norleucine, phenylalanine, serine, and valine.

Other accounts of the synthesis of alanine by the Strecker method have been reported by Heintz (88), Lubavin (89), Delépine (90), Zelinsky and Stadnikoff (91), Aschan (92), Taylor (93), and Benedict (94). However, the preparation of alanine by this method has been most satisfactorily described by Barker and Skinner (95) and Kendall and McKenzie (96). The last authors synthesized 140 to 160 gm. lots of alanine in 52 to 60 per cent of the theoretical yield.

In 1882, Lubavin (97) synthesized glycine from glyoxal (CHO CHO), ammonium eyanide and sulfuric acid. It was postulated that formaldehyde, formed as the initial product, underwent the Strecker reaction. Six years later, Jay and Curtius (98) found that ammonium cyanide reacts with two molecules of formaldehyde to give a crystalline product, methyleneaminoacetonitrile (CH₂: N·CH₂·CN), which, on acid hydrolysis, gave glycine. The preparation of this nitrile has been studied further by Bailey and co-workers (99), Johnson and Rinehart (100), Adams and Langley (101), and earlier workers referred to by these authors. In the barium hydroxide hydrolysis of the nitrile, Anslow and King (102) obtained an 84 per cent yield of glycine and Ling and Nanji (103) 90 per cent. In the preparation of large quantities of glycine by hydrolysis of the nitrile with 48 per cent hydrobromic acid, Clarke and Taylor (104) reported yields ranging from 31 to 37 per cent of the theoretical amount.

Serine was prepared from glycolaldehyde (CH₂OH·CHO) in 1902 by Fischer and Leuchs (71). Because of the difficulties encountered in the preparation of this aldehyde, either from glycolacetal [HOCH₂·CH(OC₂H₅)₂] or dihydroxymaleic acid [HOOC·C(OH):C(OH)·COOH], a modified synthesis from ethoxyacetal-dehyde (C₂H₅O·CH₂·CHO) was proposed in 1906 by Leuchs and Geiger (105). Although this has proved to be a useful method, the simplified preparation of the aldehyde directly from the ethyl ether of ethylene glycol and other improvements have been described by Dunn, Redemann, and Smith (106).

The preparation of isoleucine from d and dl-sec. valeraldehyde has been described by Ehrlich (50); leucine from isovaleraldehyde by Lubavin (89), Strecker (107), Limpricht (54), and Ehrlich (50); glutamic acid from succinic acid di-aldehyde by Keimatsu and Sugasawa (108); methionine from β -methylthiolpropionaldehyde by Barger and Coyne (59); norleucine from n-valeraldehyde by Hüfner (109); phenylalanine from phenylacetaldehyde by Erlen-

meyer and Lipp (110); and valine from isobutyraldehyde by Lipp (111).

While the described syntheses of alanine, glycine, and serine are practicable procedures, the Strecker method has been little used for the preparation of other accepted amino acids. This may be explained by the technical difficulties involved in handling the low boiling, highly toxic, anhydrous hydrocyanic acid and the low yields which often result from the use of ammonium salts and alkali cyanides as employed in the modified processes for glycine and alanine. Furthermore, it is often difficult to obtain the required aldehyde and to prevent its polymerization to an unreactive resinous state. It was shown by Eschweiler (112) and Franzen (113) that the side reaction products, iminodiacetic acid [NH(CH₂· $COOH)_2$ and trimethyleneamine tricarboxyllic acid $[N(CH_2 \cdot$ COOH)₃], may be formed in considerable quantities under certain experimental conditions in the synthesis of glycine from formaldehyde. That substances of analogous constitution may be produced in the synthesis of alanine, phenylalanine, and glutamic acid is indicated by the experiments of Passavant and Erlenmeyer (114), Erlenmeyer and Lipp (110), Dubsky (115), and Stadnikoff (116). However, an excess of ammonia and hydrocyanic acid may be used to hinder the formation of these products.

A careful study of the Strecker reaction was made in 1931 by Cocker and Lapworth (117) who synthesized glycine, alanine, and a number of unnatural amino acids in yields averaging about 70 per cent of that expected from theory. These authors recommend the exclusion of even small amounts of the alkali metals in the synthesis of the more soluble amino acids; the use of ammonium salts and alkali cyanides only for the preparation of the sparingly soluble amino acids; that barium carbonate is to be preferred to lead compounds for the elimination of ammonia from ammonium salts and for the removal of sulfuric acid because the basic lead salts of the amino acids are only slightly soluble; and that strong acids rather than strong bases be used for the hydrolysis of aminonitriles.

(2) α -Halogen Acid Synthesis. (a) Reactions with Ammonia. The general reactions by which α -halogen acids react with ammonia to form α -amino acids are given by the following equations:

$$\begin{array}{c} \text{R} \cdot \text{CH}_2 \cdot \text{COOH} \xrightarrow{\text{Br}_2 + P} \text{R} \cdot \text{CH}(\text{Br}) \cdot \text{COOH} \\ \xrightarrow{\text{2NH}_3} \text{R} \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} + \text{NH}_4 \text{Br} \end{array}$$

These reactions, first used by Perkin and Duppa (31) and Cahours (30) in 1858 for the synthesis of glycine from bromoacetic acid, have been adapted to the synthesis of alanine, aspartic acid, isoleucine, leucine, norleucine, and valine.

Additional accounts of the synthesis of glycine from bromo- or chloroacetic acid have been given by Heintz (118), Mauthner and Suida (119), Kraut (120), Drushel and Knapp (121), Curtius and Hofmann (122), Robertson (123), Boutwell and Kuick (124), and Orten and Hill (125). The amination step in this synthesis has been the subject of investigation for more than seventy-five years. With low molar ratios of ammonia to halogen acid, large amounts of iminodiacetic acid and trimethylene amine tricarboxylic acid are formed as side reaction products. Since the presence of the difficultly crystallizable ammonium salts of these strong acids in the syrupy reaction mixture interferes with the crystallization of glycine, it has been common practice to isolate the latter as its copper salt. Glycine, prepared under these conditions, is impure and the yield is only about 20 per cent of the theoretical yield. As early as 1890, Kraut recognized that the proportion of glycine to the di- and tricarboxylic acids could be increased appreciably by the use of higher ratios of ammonia to chloroacetic acid. Molal ratios as high as 20:1 were used and the yield of glycine was increased to approximately 50 per cent of the theoretical amount. By a thorough investigation of this glycine synthesis, Robertson (123) showed that the theoretical quantity of chloride ion is produced, and about 95 per cent of the total nitrogen in the reaction products is in the amino form, when the molal ratio of ammonia to chloroacetic acid is increased to 220:1. After removing the chloride ions and excess of ammonia by treatment with silver oxide, glycine was crystallized from the filtrate in 75 per cent of the theoretical yield. It was brought to a state of high purity without difficulty. Further improvements in the synthesis have been made by Orten and Hill (125) who avoid the use of expensive silver compounds by evaporating the reaction mixture to a small volume in order to remove excess of ammonia and by crystallizing the glycine from a 6:1 methyl alcohol-water solution in the presence of an equi-molar quantity of ammonium chloride. Modified procedures for the separation of glycine as the copper salt (126) and as the acety derivative (127) have been described recently.

The preparation of alanine by the action of ammonia on α -chloropropionic acid was reported in 1860 by Kolbe (128) and on

 α -bromopropionic acid by Kekulé (129) and Fischer (130). Large quantities of alanine may be readily prepared from aqueous ammonia and α -bromopropionic acid in 60:1 molal ratio. The bromoacid is conveniently prepared by the action of bromine and red phosphorous on commercial propionic acid as given by Heidelberger (131).

Analogous procedures have been described by Fischer (132) for the synthesis of leucine from α -bromoisocaproic acid; by Clark and Fittig (133), Schlebusch (134), Schmidt and Sachtleben (135), Slimmer (354), and Neuberg and Karczag (136) for valine from α -bromoisovaleric acid; and by Hüfner (109), Abderhalden and co-workers (137), Greenwald (138), Adams and Marvel (139), and Marvel and du Vigneaud (140) for norleucine from α -bromo-n-caproic acid. Yields of 65 to 75 per cent of purified products are readily obtained when the methods, described in detail by Slimmer and by Marvel and du Vigneaud, are followed.

The preparation of aspartic acid by the action of aqueous or alcoholic ammonia on monochloro- or bromosuccinic acid has been reported by Koerner and Menozzi (141), Walden and Lutz (142), and Fischer and Raske (143), but a thoroughly satisfactory synthesis has not been described. As shown by the experiments of Kekulé (129, 144), Fittig (145), Schacherl (146), Hell (147), Anschütz and Bennert (148), Perkin (149), Volhard (150), Walden (151), and McMaster and Magill (152), poor yields of impure monochloro- or bromosuccinic acid result from the action of bromine, phosphorous pentabromide and bromine, or bromine and phosphorous on succinic acid or its diethyl ester because of the formation of dibromosuccinic and maleic acids at the high temperature required for the bromination. Similar difficulties were encountered in the attempts to prepare bromo- or chlorosuccinic acid by the addition of hydrobromic or hydrochloric acid to fumaric or maleic acid. On the other hand, Kekulé and Walden found that hydrobromic acid or phosphorous pentachloride reacts smoothly with malic acid to give the desired product. Although fumaric acid is produced as a side reaction product, it appears probable that bromo- or chlorosuccinic acid can be produced satisfactorily by this method.

Except for Robertson's comprehensive study showing the favorable mass action effect of ammonia on the production of glycine from chloroacetic acid, no other comparable physico-chemical data are available. There is need for investigations to determine the in-

fluence of temperature and mass action effects of ammonia on the distribution of main and side reaction products from the synthesis of all of the accepted amino acids which can be prepared from α -halogen acids. Although Robertson concluded that the results from the synthesis of glycine at elevated temperatures were not appreciably better than those at 30°, it is not unreasonable to expect that temperature effects might be more pronounced in other cases.

The halogen acid-ammonia method is thought to be more satisfactory than any other for the synthesis of glycine, alanine, valine, and other amino acids for which the required α -halogen acids are readily obtainable. The appropriate acids for the synthesis of nor-leucine, leucine, and isoleucine may be prepared by the malonic ester method (described later in this chapter) or by the reactions (indicated below) described by Hass and Marshall (153). n-Amyl chloride, iso-amyl alcohol, and sec. butyl carbinol are the commercial substances most convenient to use for the preparation of the n-caproic, iso-caproic, and β -methyl- β -ethyl propionic acids required in the synthesis of the three leucines.

$$\begin{array}{c} \text{R} \cdot \text{CH}_2\text{OH} \xrightarrow{\text{HBr}} \text{R} \cdot \text{CH}_2\text{Br} \xrightarrow{\text{KCN}(\text{C}_2\text{H}_5\text{OH})} \\ \text{R} \cdot \text{CH}_2 \cdot \text{CN} \\ \\ \xrightarrow{\text{2H}_2\text{O}(\text{H}_2\text{SO}_4)} \xrightarrow{\text{R} \cdot \text{CH}_2 \cdot \text{COOH} + \text{NH}_4\text{Br}} \end{array}$$

(b) Reactions with Ammonium Carbonate. Ammonium carbonate was first used as an aminating agent in 1883 by Nencki (154) who synthesized glycine by heating crystalline chloroacetic acid and dry ammonium carbonate to 130°. Glycine was isolated as the copper salt in 20 per cent of the theoretical yield. Approximately the same results were reported five years later by Mauthner and Suida (119) who maintained a solution of chloroacetic acid, sodium carbonate, and an excess of ammonia at the boiling temperature for about 8 hours. In 1902, Slimmer (354) synthesized value in 70 per cent of the theoretical yield by heating 500 gm. of α -bromoisovaleric acid with 1500 gm. of saturated aqueous ammonia and 500 gm. of ammonium carbonate for 8 hours at 100° in an autoclave. Although Robertson (123) concluded that ammonium carbonate is inferior to ammonia for the synthesis of glycine, Cheronis (155) made the striking discovery that the yield of glycine which is obtained by heating ammonium carbonate and chloroacetic acid in a molal ratio of 4:1 at 60 to 65° is as good as that obtained from ammonia and chloroacetic acid in the ratio of 60:1 at room temperature.

As an explanation of these results, it might be assumed that the relatively high temperature is more favorable to the production of glycine than of the side reaction products, iminodiacetic acid and trimethylene amine tricarboxylic acid. However, it seems probable that the hydroxide, ammonium, carbonate, bicarbonate, or carbamate ions are, at least, partly responsible for this effect. While the Cheronis method is considered to be superior to any other for the synthesis of glycine, it has been applied less satisfactorily to the preparation of other amino acids in the author's laboratory.

(c) Reactions with Potassium phthalimide. In 1888, Goedeckemeyer (156) described a synthesis of glycine from potassium phthalimide and chloroacetic ester. A year later, Gabriel and Kroseberg (157) made a detailed study of this method. The steps in the synthesis are shown by the following equations:

The yields of products in the first two steps of the synthesis were 97 and 85.5 per cent of the theoretical. Glycine was also prepared by the direct acid hydrolysis of phthalyl glycine ethyl ester in a closed tube at 200°. The over-all yield was 93 per cent of the theoretical amount. Although only small quantities of glycine could be prepared by this method, advantages, not possessed by other procedures available at that time, were freedom from trouble-some side reaction products, high percentage yield, and high purity of product. While the phthalimide reactions are no longer used for the synthesis of glycine, they have been adapted to the preparation of other amino acids as described later in this chapter.

(3) Malonic Ester Synthesis. (a) Reactions of Malonic Ester with Halogen Compounds. These reactions are represented by the following equations:

Alanine, histidine, isoleucine, leucine, lysine, methionine, norleucine, hydroxyproline, phenylalanine, proline, and valine have been synthesized by this method. Alanine was prepared by Lutz (158) with methyl bromide as the alkylating agent; histidine, by Pyman (159) from 4-chloromethylglyoxaline and sodium chloromalonic ester. [The latter compound was prepared by Conrad's (160) method]; isoleucine by Romburgh (161), Brasch and Friedmann (162), Ehrlich (51), and Abderhalden and Zeisset (53) by the use of sec. butyl iodide; leucine by Fischer and Schmitz (163) by the use of isobutyl bromide. [The intermediates, isobutylmalonic ester and isobutylmalonic acid, were previously described by Bischoff (164) and Guthzeit (165)]; lysine by Fischer and Weigert (56) from γ-chlorobutyronitrile. [The intermediate γ-cyanopropylpropylmalonic ester was prepared ten years earlier by Blank (166)]; methionine by Windus and Marvel (167) by the use of methylthiolethyl chloride. [Improvements in this synthesis have been described by Schmidt and co-workers (168)]; norleucine by Adams and Marvel (139) by using n-butyl bromide. [The intermediate n-butylmalonic ester was described earlier by Bischoff (164) and Adams and Kamm (169)]; hydroxyproline by Traube and Lehmann (170) and Leuchs (41) by the use of epichlorohydrin; phenylalanine by Fischer (171) and Leuchs (172) with benzyl chloride. [The intermediate benzylmalonic ester was prepared by Conrad (173) many years earlier]; and proline by Willstätter (69) and Willstätter and Ettlinger (174) by using trimethylene bromide. The intermediate γ -bromopropyl malonic ester was described later by Leuchs (172). No account of the synthesis of valine by this method has been given, although the intermediate isopropylmalonic ester has been prepared by Bischoff (164) and Marvel and du Vigneaud (175). Yields of ester, ranging from 70 to 75 per cent of the theoretical amount, were reported by the latter authors. The preparation of α -bromoisovaleric acid from isovaleric acid was also described by Marvel and du Vigneaud.

Although the malonic ester method is widely used for the synthesis of isoleucine, leucine, methionine, norleucine, and phenylalanine, its usefulness is limited by the numerous steps, the relatively low over-all yields, the unavailability of certain halides, and the ready formation of side reaction products. In addition to the monoalkyl or aryl malonic ester derivatives, considerable quantities of the di-substituted products may be formed. The probable mechanism of these reactions has been discussed by Dunn and coworkers (176). However, it is well known that the yield of disubstituted esters is much lower with aliphatic than with aromatic halides. Furthermore, the yield of monosubstituted aryl esters may be considerably increased by the mass action effect of excess of malonic ester. In their synthesis of proline, Willstätter and Ettlinger were able to prepare γ-bromopropylmalonic ester in only 28 per cent yield owing to the formation of the side reaction products, cyclobutane dicarbonic acid ester and pentane tetracarbonic acid ester. With an excess of malonic ester, Leuchs found that the yield of monosubstituted ester was increased to about 38 per cent of the theoretical.

(b) Reactions of Phthalimidomalonic Ester with Halogen Compounds. The equations for the synthesis of amino acids by this method are given below:

$$\begin{array}{c|c} CO(OC_2H_5) & CO(OC_2H_5) \\ \hline CH_2 & CHBr \\ \hline CO(OC_2H_5) & CO(OC_2H_5) \\ \hline \\ CO(OC_2H_5) & CO(OC_2H_5) \\ \hline \\ (Malonic \ ester) & (Bromomalonic \ ester) \\ \hline \end{array}$$

The foregoing modification of Gabriel's phthalimide synthesis was adapted by Sörensen (177–179), from 1903 to 1908, to the preparation of phenylalanine, proline, and the unnatural amino acids, ornithine and α -aminoadipic acid. Preliminary experiments on the synthesis of lysine from δ -bromobutylphthalimide were described (reference 177, page 60) by Sörensen, but he stated later that "I have not succeeded in elaborating the reduction process in such a manner that the yield of lysine seems to me to be sufficient, and, therefore, I have not published more about this question than this preliminary paper" (180).

The halogen compounds, benzyl chloride and trimethylene bromide, were used by Sörensen in his syntheses of phenylalanine and proline, respectively. The following additional amino acid syntheses by Sörensen's method have been reported: tyrosine by Stephen and Weizmann (181) from anisyl bromide (CH₃O·C₆H₄·CH₂Br), serine by Mitra (182) from monochlorodimethyl ether (ClCH₂·OCH₃), methionine by Barger and Weichselbaum (183) from β -chloroethylmethyl sulfide, and aspartic acid by Dunn and Smart (184) from chloroacetic ester. The last authors found that glycine is produced in 20 per cent yield by the direct alkaline hydrolysis of phthalimidomalonic ester, but that stable condensation products leading to the attempted synthesis of glutamic acid could not be produced from any of the halogen compounds: ethylene bromide, ethylene chloride, β -chloropropionic ester.

By making use of the stable yellow solid, sodium (or potassium) phthalimidomalonic ester, disubstituted derivatives cannot be formed. However, the preparation and purification of sodium phthalimidomalonic ester from phthalimidomalonic ester, sodium, and alcohol by Sörensen's original method is tedious. Later workers found that this reaction may be carried out more conveniently using dry toluene, xylene, or an ether-alcohol mixture. Also, the decomposition of the alkyl or aryl phthalimidomalonic ester derivative may be carried out more effectively with hydrobromic acid or hydrazine (185) than with hydrochloric acid or the two step alkaline-acid procedure employed by Sörensen.

(c) Reactions of Aminomalonic Ester and Derivatives with Halogen Compounds. The following equations represent the synthesis of amino acids by this method:

The preparation of aminomalonic acid and its decomposition to glycine and carbon dioxide are described in papers by Baeyer (186), Lange (187), Ruhemann and Orten (188), and Piloty and Finckh (189). Isonitrosomalonic ester was first prepared by Conrad and Bischoff (190) who treated sodium malonic ester with nitrous acid. However, many essential experimental details were omitted in their description of the method. Nitrosation by means of methyl nitrite in absolute alcohol was described by Bouveault and Wahl (191) and Putochin (192). Butyl nitrite, prepared by the method of Noyes (193), has been used satisfactorily by Redemann (194). In 1928, Locquin and Cherchez (195) stated that isonitrosomalonic ester was prepared in 90 per cent of the theoretical yield by the action of an aqueous solution of sodium nitrite on malonic

ester in acetic anhydride. Two years later, Cherchez (196) reported 80 to 90 per cent yields of the ester by treating malonic ester in glacial acetic acid with aqueous sodium nitrite. However, Dunn and co-workers (197) were not able to duplicate these results.

Locquin and Cherchez (198) were not able to prepare aminomalonic ester by the reduction of isonitrosomalonic ester with magnesium amalgam, but these authors (195), as well as Piloty and Neresheimer (199), Putochin (192), and Dunn and co-workers (197), found that aluminum amalgam is a satisfactory reducing agent. As pointed out by the last authors, active aluminum is required for the preparation of an effective reducing amalgam. Reduction by means of hydrogen sulfide in alkaline solution was found by Johnson and Nicolet (200) to be impracticable for the preparation of relatively large quantities of isonitrosomalonic ester owing to the hydrolysis of the latter. The method described in 1934 by Levene and Schormüller (201) for effecting reduction by means of the Raney (nickel) catalyst has been found by Redemann (194) to give excellent results.

While the reduction of isonitrosomalonic ester to glycine by means of zinc and 80 per cent acetic acid was accomplished in 1909 by Conrad and Schulze (202), the first complete synthesis of an amino acid by the reactions shown above was reported by Putochin (192) in 1923. A small quantity of proline was prepared from the reaction of aminomalonic ester with trimethylene bromide. A few years later, Keimatsu and Kato (203) synthesized aspartic acid by this method using chloroacetic ester, while Locquin and Cherchez (195) prepared leucine and phenylalanine from the corresponding halogen compounds. In 1931, Dunn and co-workers (197) described the synthesis of glutamic acid from β -chloropropionic ester and the hitherto unknown benzoylaminomalonic ester.

Syntheses of amino acids through the agency of aminomalonic ester or its hydrochloride are unsatisfactory because of the instability of the free base, the hygroscopic qualities of the salt, and the reactions which halogen compounds undergo with the amino, as well as the methylene, group to form imino derivatives. Many of these difficulties are eliminated by the use of benzoylaminomalonic ester. The general applicability of this method has been demonstrated by Redemann (194) in the satisfactory synthesis of aspartic acid, leucine, phenylalanine, and valine. It is expected that this method can be applied successfully to the synthesis of a dozen or more amino acids.

(d) Reactions of Potassium Ethylmalonate and Derivatives with Hydrazine. These reactions (shown below) were utilized by Curtius

$$\begin{array}{c} \operatorname{CO(OC_2H_5)} & \operatorname{COOK} \\ \mid & \operatorname{KOH} + \operatorname{N_2H_4} \\ \mid & \operatorname{CHR} \\ \hline & (\operatorname{C_2H_5OH}) \\ \mid & \operatorname{CO(NH \cdot NH_2)} \\ (\operatorname{Malonic ester} & (\operatorname{Potassium malonic} \\ \operatorname{or derivative}) & \operatorname{hydrazide}) \\ \end{array}$$

$$\begin{array}{c} \operatorname{COOH} \\ \mid & \operatorname{COOH} \\ \mid & \operatorname{COOH} \\ \mid & \operatorname{CHR} \\ \mid & \operatorname{CHR} \\ \mid & \operatorname{CHR} \\ \mid & \operatorname{CHR} \\ \mid & \operatorname{CAmino} \\ & \operatorname{mediate}) \\ \end{array}$$

and co-workers (204, 205) for the synthesis of alanine, glycine, phenylalanine, and valine. Although the yields of aliphatic amino acids, isolated as their ethyl ester hydrochlorides, were as high as 80 per cent of the theoretical yield, difficulties were encountered in the synthesis of phenylalanine owing to the instability of the water insoluble benzyl malonic acid hydrazide and the formation of 3,6-dibenzyl-2,5-diketopiperazine and the anhydride of α -carbamino phenylpropionic acid. Although Curtius (206) stated that aspartic acid, glutamic acid, leucine, and tyrosine may be prepared by these general reactions, no other amino acid syntheses have been reported.

(4) Condensation with Aldehydes. (a) Reactions with Hydantoins. Equations for these reactions are given below:

$$\begin{array}{c|c} NH-CO & NH-CO \\ \hline CO & \hline \\ CO & \hline \\ CH_3 \cdot COONa, CH_3 \cdot COOH, (CH_3 \cdot CO)_2O \\ \hline NH-CH_2 & NH-C = CHR \\ (Hydantoin) & (4-R-alhydantoin) \\ \hline NH-CO & \hline \\ CO & \hline \\ NH-CH_2 \cdot R \\ (4-R-ylhydantoin) & (Amino acid hydroiodide) \\ \hline \\ NH-CH \cdot CH_2 \cdot R \\ (4-R-ylhydantoin) & (Amino acid hydroiodide) \\ \hline \end{array}$$

The synthesis of leucine from 4-isobutylhydantoin was reported by Pinner and Spilker (207) in 1889. However, subsequent use of the hydantoin method has been limited to reactions with the aryl halides benzaldehyde, anisaldehyde (CH₃OC₆H₄CHO), and β-indole aldehyde

in the synthesis of phenylalanine, tyrosine, and tryptophane respectively. The synthesis of tryptophane was described in 1922 by Majima (208), while the preparation of phenylalanine and tyrosine was reported earlier by Wheeler and Hoffman (209) and Johnson and collaborators (210–213).

In general, the hydantoin reactions proceed smoothly and lead to good yields of phenylalanine and tyrosine. However, relatively low yields of tryptophane were obtained by Majima owing to difficulties in the preparation of β -indole aldehyde and β -indolalhydantoin. While Majima reported a 68 per cent yield of ω -hydantoylskatole from the reduction of β -indolalhydantoin with sodium amalgam, these results are somewhat difficult to explain in view of the ready reduction of the indole double bond by strong reducing agents. R-alhydantoins may be effectively reduced by tin and hydrochloric acid, particularly when an excess of stannic chloride, which hinders the separation of the reduced product, is avoided. Simultaneous reduction and hydrolysis of the R-alhydantoin are accomplished satisfactorily by the use of hydriodic acid and red phosphorous.

The relatively large quantities of hydantoin required for the synthesis of amino acids by this method may be readily prepared by the acid hydrolysis of ethylhydantoate (211). The latter compound may be synthesized from glycine ethyl ester and potassium cyanate by the method of Harries and Weiss (214), while potassium cyanate may be prepared from potassium ferrocyanide and potassium dichromate as described by Erdmann (215).

On account of the high cost of potassium cyanate, Johnson and O'Brien (213) substituted potassium thiocyanate in a series of reactions leading to the synthesis of phenylalanine. The intermediate substances, 2-thio-3-benzoylhydantoin, 2-thio-3-benzoyl-4-benzalhydantoin, 2-thio-4-benzalhydantoin, and 2-thio-4-benzylhydantoin, were formed under conditions similar to those described for the

corresponding hydantoin derivatives. Johnson and O'Brien reported almost a quantitative yield of phenylalanine by this method, but it has not been possible to duplicate these results in the author's laboratory.

(b) Reactions with Diketopiperazine (Glycine Anhydride). Equations for these reactions are given below:

The diketopiperazine method, proposed in 1921 by Sasaki (216), was adapted by this author to the synthesis of phenylalanine and tyrosine. Benzaldehyde and anisaldehyde were condensed with diketopiperazine to give 3:6-dibenzal-2:5-diketopiperazine and 3:6-dianisal-2:5-diketopiperazine in yields of 62 and 54 per cent of the theoretical respectively. These intermediates were reduced with hydriodic acid and red phosphorous and the resulting products were hydrolyzed to phenylalanine and tyrosine in 83 and 90 per cent of the theoretical yields.

Glycine anhydride may be conveniently prepared by Fischer's (217) method. Glycine ethyl ester is allowed to react at room temperature with water in order to minimize the formation of side reaction products. By this method Fischer synthesized 100 gm. of glycine anhydride from 560 gm. of glycine ethyl ester hydrochloride. Although Dickinson and Marshall (218) obtained only 36 per cent of the theoretical yield, Fischer's results have been duplicated by Stoddard in the author's laboratory. Approximately 60 per cent of the theoretical yields of glycine anhydride have been prepared by a modification of the method described in 1910 by Pribram (219).

While both phenylalanine and tyrosine may be synthesized in

quantity and in good yields by Sasaki's excellent method, Redemann's (194) attempts to adapt this procedure to the preparation of tryptophane were unsuccessful owing to the insolubility of the acetylated 3:6-di-indolal-2:5-diketopiperazine and his inability to effect selective reduction of this compound. No other syntheses of amino acids by Sasaki's method have been reported.

(c) Reactions with Hippuric Acid (Azlactone Method). These reactions (shown below) were first investigated by Plöchl (220) in 1883. One year later, this author (221) prepared phenylalanine from benzaldehyde, hippuric acid, and acetic anhydride. However, interpretations of the

$$\begin{array}{c} \text{COOH} & \text{R} \cdot \text{CH} = \text{C} - \text{CO} \\ & \text{CH}_2 & \text{CH}_3 \cdot \text{COONa} + (\text{CH}_3 \cdot \text{CO})_2\text{O} & \text{H}_2\text{O}(\text{NaOH}) \\ & \text{NH} \cdot \text{CO} \cdot \text{C}_6\text{H}_5 & \text{N} = \text{C} \cdot \text{C}_6\text{H}_5 \\ & \text{(Hippuric acid lactimide (or azlactone)} & & & & \\ & \text{R} \cdot \text{CH} = \text{C} \cdot \text{COOH} & \text{HI} + \text{P} & \text{R} \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{COOH} \\ & \text{NH} \cdot \text{CO} \cdot \text{C}_6\text{H}_5 & \text{CH}_3 \cdot \text{COOH} & \text{NH} \cdot \text{CO} \cdot \text{C}_6\text{H}_5 \\ & (\alpha \cdot \text{Benzoylamino} - & (\alpha \cdot \text{Benzoylamino} - \\ & \beta \cdot \text{R} - \text{acrylic acid}) & \beta \cdot \text{R} - \text{propionic acid} \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & & \\ & & \\ & & & \\ & & \\ & & \\ & & & \\ & \\ & & \\ & & \\ & & \\ & & \\ & & \\ & & \\ & & \\ & & \\ & & \\ & & \\ & & \\ &$$

mechanism of these reactions and improvements in the phenylalanine synthesis were described by Erlenmeyer and Kunlin (222–228) in a series of papers published from 1892 to 1904. In more recent times, the synthesis has been further improved by Harington and McCartney (229), Lamb and Robson (230), and Gillespie and Snyder (231).

In addition to phenylalanine, Erlenmeyer's azlactone method has been used for the synthesis of the following amino acids: leucine from isobutyraldehyde by Erlenmeyer and Kunlin (232), tyrosine from p-hydroxybenzaldehyde by Erlenmeyer and Halsey (233) and Fischer (234), tyrosine from p-methoxybenzaldehyde by Harington and McCartney (229) and Lamb and Robson (230), histidine from glyoxaline formaldehyde by Pyman (235), tryptophane by Ellinger and Flamand (77) from β -indole aldehyde, and

thyroxine from 3:5-diiodo-4-(4'-methoxyphenoxy) benzaldehyde by Harington and Barger (72) and Harington and McCartney (229).

The usefulness of the azlactone method for the synthesis of aromatic amino acids is generally recognized. When hydriodic acid and red phosphorous are employed in place of the original reducing agent, sodium amalgam, and hydriodic acid is used as the hydrolyzing agent, instead of hydrochloric acid, each of the four main reactions proceeds smoothly and the yields of intermediate and final products are relatively high.

(5) α-Keto Acid Reactions. (a) Reduction and Amination. Five amino acids have been prepared by the following general reactions: $R \cdot CO \cdot COOH + H_2 + NH_3 + catalyst \rightarrow R \cdot CH(NH_2) \cdot COOH + H_4O.$ Löb (236) converted glyoxylic acid, prepared by the electrolytic reduction of oxalic acid, to hydroxyaminoacetic acid [NH₂CH(OH)COOH] and the latter to glycine. Alanine was prepared from pyruvic acid (CH₃COCOOH) by Aubel and Bourguel (237), while aspartic acid, glutamic acid, and phenylalanine were synthesized from oxaloacetic acid (HOOC · CH4 · CO · COOH), α-ketoglutaric acid (HOOC·CH₄·CH₂·CO·COOH), and phenylpyruvic acid (C₆H₅·CH₂·CO·COOH), respectively, by Knoop and Oesterlin (238, 239). In the experiments by the last authors, colloidal platinum and palladium were used to catalyze the reductions. With phenylalanine, the yield was more than 60 per cent of the theoretical but it was only 23 to 32 per cent in the case of aspartic and glutamic acids. However, only 0.8 to 3.2 gm. of these amino acids were prepared.

The following type reactions have been used for the synthesis of three amino acids:

Erlenmeyer (227) and Erlenmeyer and Kunlin (227, 232) synthe-

sized phenylalanine from phenylpyruvic acid through the intermediate phenylacetylaminophenylalanine [C₆H₅·CH₂·CH(COOH)·NHCO·CH₂·C₆H₅]. Alanine was prepared by De Jong (240) from pyruvic acid through acetylalanine, while glycine was synthesized by Erlenmeyer and Kunlin (241) from glyoxylic acid through formylglycine, and by Erlenmeyer (242) from a mixture of glyoxylic and pyruvic acids through the intermediate acetylglycine.

- (b) Reduction of Oximes. The general reactions: R · CO · COOH $+ NH_2OH \cdot HCl \rightarrow R \cdot C(:NOH) \cdot COOH \xrightarrow{H_2} R \cdot CH(NH_2) \cdot COOH$ +H₂O have been adapted to the synthesis of three amino acids. Piutti (23) prepared aspartic acid by the reduction of succinic ester α -oxime $[(C_2H_5O)OC \cdot C(:NOH) \cdot CH_2 \cdot CO(OC_2H_5)]$. Isoleucine was synthesized by Bouveault and Locquin (243) who hydrogenated the oxime of methyl ethyl pyruvic ester [CH3 CH2. CH(CH₃)·C(:NOH)·CO(OC₂H₅)] while phenylpyruvic acid oxime [C₆H₅·CH₂·C(:NOH) ·COOH] was reduced to phenylalanine by Erlenmeyer (224, 244, 245), Posner (246), and Knoop and Hoessli (247). Tin and hydrochloric acid, zinc and hydrochloric acid, aluminum amalgam, and sodium amalgam were used to reduce the oximes. The quantities of oximes used or of amino acids prepared were not reported except by Bouveault and Locquin who obtained 60 to 70 per cent of the theoretical yields of isoleucine ester.
- (c) Reduction of Hydrazones. The general reactions: $R \cdot CO \cdot COOH + HCl \cdot NH_2 \cdot NH \cdot C_6H_5 \rightarrow R \cdot C (:N \cdot NH \cdot C_6H_5) \cdot COOH \xrightarrow{2H_2} R \cdot CH(NH_2) \cdot COOH + NH_2 \cdot C_6H_5$ were applied by Fischer and Groh (248) to the synthesis of alanine. These authors prepared 2.75 gm. (55 per cent of theoretical yield) of alanine from pyruvic acid hydrazone using aluminum amalgam as the reducing agent.

The synthesis of amino acids by the foregoing reduction methods is of limited importance owing to the unavailability of the required α -ketoacids, technical difficulties in effecting reductions and in isolating the amino acids, and the existence of more convenient methods.

(6) Unsaturated Acid Reactions. Only aspartic acid of the accepted amino acids has been prepared by this method. In 1896, Tanatar (249) synthesized aspartic acid by the decomposition of the calcium salt of fumaric acid oxime [HOOC·C(:NOH)·CH₂·COOH], but the yield was not given. Philippi and Uhl (250) obtained 0.2 gm. of aspartic acid by the acid hydrolysis of 1-amino-1. 1-diethylcarboxy-2, 2-diethylcarboxyethane

$$(C_2H_5O)OC \qquad CO(OC_2H_5)$$

$$CH-C\cdot NH_2$$

$$(C_2H_5O)OC \qquad CO(OC_2H_5)$$

formed by the action of ammonia on 1, 1-diethylcarboxy-2, 2-diethylcarboxyethylene

$$(C_2H_5O)OC \\ C = C \\ (C_2H_5O)OC \\ CO(OC_2H_5)$$

but this method is only of theoretical interest.

The direct addition of ammonia to maleic and fumaric acids was investigated by Engel (251) and Stadnikoff (252). When the amination was conducted at 140 to 150° in aqueous or alcoholic solution, Engel obtained 30 to 35 per cent of the theoretical yield of aspartic acid. However, the quantity of amino acid synthesized and the purity of the product were not given. Stadnikoff isolated 3 gm. of diethyl aspartate, 2 gm. of diethyl iminosuccinate, and a small amount of an unidentified third substance by the fractional distillation in vacuo of the esterified products resulting from the reaction of 20 gm. of fumaric acid and an excess of ammonia. By the addition of ammonia to fumaric ester, Koerner and Menozzi (253) isolated from the reaction products some diethyl aspartate and a small amount of a crystalline substance which they believed to be aspartimide,

Later, Fischer and Koenigs (254) showed that this substance is more probably the isomeric 3:6 diacetamide-2:5-diketopiperazine (the diamide of the diketopiperazine of aspartic acid)

$$\begin{array}{c} & O \\ H & \parallel \\ NH_2 \cdot CO \cdot CH_2 - C - C - NH \\ & \parallel \\ N - C - CH \cdot CH_2 \cdot CO \cdot NH_2 \\ H & \parallel \\ O \end{array}$$

A convenient and practicable synthesis of aspartic acid was de-

scribed in 1933 by Dunn and Fox (255). The crystalline diketopiperazine diacetamide was the principal product formed when alcoholic ammonia was allowed to react with diethyl fumarate for 24 hours at 100° and 6 atmospheres. This intermediate was hydrolyzed with alkali and aspartic acid was isolated as the trihydrated copper salt. A 78 gm. lot (59 per cent of the theoretical yield) of analytically pure aspartic acid was prepared.

(7) Racemization. The effectiveness of barium hydroxide as a racemizing agent was demonstrated by Schulze and co-workers (66, 256). Tyrosine, leucine, phenylalanine, and glutamic acid were inactivated by heating conglutin for 4 days at 150 to 160° with a saturated solution of barium hydroxide. Inactive leucine was prepared by heating the active form for three days under the same conditions. In more recent times sodium hydroxide, potassium hydroxide, strontium hydroxide, calcium hydroxide, barium hydroxide, lead oxide, hydrochloric acid, hydriodic acid, and sulfuric acid have been employed as racemizing agents. However, barium hydroxide is preferred because it is readily removed from the reaction mixture as insoluble barium sulfate. In racemizing different amino acids with this base, times ranging from 5 to 72 hours and temperatures from 150 to 180° have been employed by various investigators. Among the amino acids which have been racemized with barium hydroxide or other alkalies are alanine (257), glutamic acid (66, 258, 259), histidine (260), hydroxyproline (43), leucine (66, 256, 261), lysine (262, 263), methionine (58), phenylalanine (66), serine (143), and tyrosine (66, 256).

In the racemization of amino acids with alkalies, serious decomposition often results with the formation of ammonia; amines; salts of carbonic, oxalic, pyruvic, lactic, hydrogen sulfide, and other acids; and other products. Hence, the yields of inactive amino acids may be very low and the racemized products difficult to isolate and purify. Arginine, aspartic acid, cystine, lysine, and glutamic acid are particularly sensitive to the action of hot alkalies. Cystine may be completely destroyed, while glutamic acid may be transformed in part to a-pyrrolidone carboxylic acid. Fischer and co-workers (258) found that inactive glutamic acid may be separated from the more soluble α-pyrrolidone carboxylic acid and obtained in a 50 per cent yield by repeated crystallization from water. It seems probable that this yield could be increased somewhat by subsequent treatment with strong acids which have been shown (264-266) to hydrolyze α-pyrrolidone carboxylic acid to glutamic acid.

Racemization of amino acids with acids was first reported by Michael and Wing (267) in 1884. Inactive aspartic acid, identical with that synthesized by Dessaignes (87), was prepared by heating a water solution of active aspartic acid hydrochloride to 170 to 180°. A year later, these investigators (268) found that natural aspartic acid was not racemized when heated in dilute hydrochloric acid at temperatures from 100 to 160°, and that complex decomposition products were formed at 200°. These results were confirmed by Vallée (269). Riesser (270) obtained 50 to 60 per cent of the theoretical yield of the inactive product by heating d-arginine carbonate with sulfuric acid for 33 hours at 160 to 180°. Siegfried (271) inactivated lysine by heating the dihydrochloride with 24 per cent hydrochloric acid for 15 hours at 160 to 170°.

The racemization of cystine presents unusual difficulties. This amino acid may be completely decomposed by treatment with alkalies. By long continued exposure to hot concentrated solutions of strong acids, complete racemization may be effected, but considerable quantities of hydrogen sulfide, mercaptans, and other decomposition products are formed. In the original investigation of this problem Mörner (272) found that natural cystine is only partially racemized by heating it for 109 hours in 10 per cent hydrochloric acid at 100°. A few years later, Neuberg and Mayer (273) prepared inactive cystine in 50 per cent of the theoretical yield by heating *l*-cystine with 15–20 times the quantity of hydrochloric acid (sp. gr. 1.124) for 12 to 15 hours at 165°. The physical and chemical effects of both acids and alkalies have been investigated in recent times by Gortner (274, 275), Clarke (276), Andrews (277), and Toennies (278) and their co-workers. In 1935, Toennies and Elliott (279) found, on boiling 24 gm. of l-cystine for 46 hours with 80 mL. of 54 per cent sulfuric acid, that the optical rotation of the solution was less than 0.2 per cent of the original value. By repeated fractionation of the 18.5 gm. of crude inactive cystine, 4 gm. of dl- and 8.5 gm. of meso cystine were isolated.

In the racemization of serine by alkalies, Daft and Coghill (280) found that ammonia, glycine, alanine, and salts of oxalic, lactic, and pyruvic acid were formed as decomposition products.

A new racemization technique with acetic anhydride has been devised by du Vigneaud and co-workers (281, 282). dl-Acetylated histidine, methionine, tryptophane, cystine, glutamic acid, arginine, tyrosine, and phenylalanine were formed by treating these amino acids with sodium hydroxide or pyridine and excess acetic anhydride. It was shown further that proline is not racemized

under these conditions, that the addition of free base is not required for the racemization of acetyl-d-arginine, and that inactivation of the amphoteric amino acids occurs only in slightly basic solution. The transitory formation of amino acid lactones, as indicated below, was thought to be the most probable explanation for the observed reactions. It was noted that proline is incapable of existence in the azlactone form.

B. Amino Acid Syntheses

(1) Alanine. From 100 to 200 gm. lots of alanine are most conveniently prepared from acetaldehyde, ammonium chloride, and sodium cyanide (96), or from α -bromopropionic acid (synthesized from commercial propionic acid) and concentrated ammonia in a molal ratio of approximately 60:1 (130). In either case the yield of recrystallized product, free from ammonia and halogen ions, ranges from 50 to 60 per cent of the theoretical yield. Both reactions are carried out at atmospheric temperature and pressure. Halogen ions, as well as free and combined ammonia, may be removed by treatment with lead oxide, lead carbonate, or silver oxide. However, the preferred method is to remove the excess of ammonia by evaporation and to crystallize the amino acid from a 6:1 watermethyl alcohol solution in the presence of an equimolal quantity of ammonium halide (125). Cocker and Lapworth (117) reported a 71.8 per cent of the theoretical yield of alanine from the reaction of acetaldehyde with excess of ammonia and hydrocyanic acid. Barium carbonate was utilized for the removal of sulfuric acid and ammonia.

Other methods for the synthesis of alanine, which are of theoretical interest but of little practical value, are: reduction of the silver salt of α -nitrosopropionic acid [CH₃·CH(NO)·COOAg] (283); thermal decomposition of cysteine in dilute aqueous solution (284); reduction of serine (71), α -amino- β -chloropropionic acid (285), and cystine (286); fission of 5-methyl-4-glyoxalone (287); and hydrolysis of hydropyruvinureid (288), α -azidopropionic ester (204), and

intermediates formed by the action of sodium nitrite and hydrochloric acid on potassium methylmalonic acid hydrazide (205).

(2) Arginine. Schulze and Winterstein (18) prepared approximately 1 gm. of arginine, isolated as the copper salt and the nitrate, by the evaporation of cyanamide and ornithine over sulfuric acid for a period of three weeks. The yield was 37 per cent of the theoretical. However, the ornithine for this synthesis was prepared from arginine through ornithuric acid (dibenzoylornithine). The only complete synthesis of arginine is that described by Sörensen and co-workers (20, 289). Chemical equations which show these reactions are given below:

OC₂H₅

OC₀

ONa

+BrCH₂· CH₂· CH₂· N

OC

CO (OC₂H₅)

(Sodium phthalimidomalonic ester)

CO(OC₂H₅)

CO

NCCH₂· CH₂· CH₂· N

CO

NCCH₂· CH₂· CH₂· N

COONa

4NaOH

NaOOC · C₆H₄· CO · NHCCH₂· CH₂· CH₂· NH · CO · C₆H₄COONa

COONa

(Tetra sodium salt of
$$\gamma$$
-phthaloylaminopropylphthaloylaminomalonic acid)

COOH

2H₂O(4HCl)

COOH

COO

$$\begin{array}{c} C_{6}H_{5}\cdot CO\cdot NH\cdot CH_{2}\cdot CH_{2}\cdot CH_{2}\cdot CH(NH\cdot CO\cdot C_{6}H_{5})\cdot COOH \xrightarrow{\qquad\qquad} \\ \qquad \qquad (Dibenzoylornithine) & CN\cdot NH_{2} \\ NH_{2}\cdot CH_{2}\cdot CH_{2}\cdot CH_{2}\cdot CH(NH\cdot CO\cdot C_{6}H_{5})\cdot COOH \xrightarrow{\qquad\qquad} \\ \qquad (\alpha\text{-Benzoylamino-}\delta\text{-amino-}n\text{-valeric acid}) & (Cyanamide) \\ NH_{2}\cdot C(:NH)\cdot NH\cdot CH_{2}\cdot CH_{2}\cdot CH(NH\cdot CO\cdot C_{6}H_{5})\cdot COOH \xrightarrow{\qquad\qquad} \\ \qquad (\alpha\text{-Benzoylamino-}\delta\text{-guanido-}n\text{-valeric acid}) \\ NH_{2}\cdot C(:NH)\cdot NH\cdot CH_{2}\cdot CH_{2}\cdot CH_{2}\cdot CH(NH_{2})\cdot COOH \\ \qquad (\alpha\text{-Amino-}\delta\text{-guanido-}n\text{-valeric acid or arginine}) \end{array}$$

Approximately 4 gm. of arginine nitrate were synthesized in an over-all yield of 12.5 per cent, calculated from ornithuric acid.

Ornithine, required for the synthesis of arginine, has been isolated from arginine hydrolysates as the dibenzoyl derivative by Karrer and Ehrenstein (290), and as the dihydrochloride by Bergmann and Zervas (291). Riesser (270) prepared ornithine as the picrate by Sörensen's method. Fischer (292) synthesized ornithuric acid by the following reactions:

CO
$$CO(OC_2H_5)$$

N·K+BrCH₂·CH₂·CH₂Br+CH₂

CO $CO(OC_2H_5)$

(Potassium (Trimethyl- (Malonic ester))

CO $CO(OC_2H_5)$

Br₂

N·CH₂·CH₂·CH₂·CH

(HCCl₃)

(γ -Phthalimidopropylmalonic ester)

CO $CO(OC_2H_5)$

N·CH₂·CH₂·CH₂·CBr

(HBr)

CO $CO(OC_2H_5)$

(γ -Phthalimidopropyl bromomalonic ester)

CO $CO(OC_2H_5)$

N·CH₂·CH₂·CH₂·CBr

(HBr)

CO $CO(OC_2H_5)$

(γ -Phthalimidopropyl bromomalonic ester)

$$\begin{array}{c} \text{CC}_{6}\text{H}_{5} \cdot \text{COCl}(4\text{NaOH}) \\ \text{COOH} & \text{COOH} \\ \text{(Ornithine dihydrochloride)} \end{array}$$

 $\begin{array}{c} C_6H_5\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH\cdot CO\cdot C_6H_5)\cdot COOH\\ \\ (Ornithuric\ acid) \end{array}$

Ornithine dihydrochloride was not isolated in the pure state. However, the over-all yield from the other reactions, calculated from γ -phthalimidopropylmalonic ester, was approximately 28 per cent. This ester, first prepared in 1890 by Gabriel (293), was synthesized in 80 per cent of the theoretical yield by Gabriel and Aschan (294).

By treating β-vinylacrylic acid (CH₂:CH·CH:CH·COOH) with ammonia for 20 hours at 150°, Fischer and Raske (295) obtained 70 to 80 per cent of the theoretical yield of ornithine dipicrate. Starting with piperidine, Fischer and Zemplén (296) prepared ornithuric acid in approximately 19 per cent over-all yield by the following reactions:

 $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH \xrightarrow{Br_2+P}$ (δ -Benzoylamino-n-valeric acid)

 $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(Br) \cdot COOH \xrightarrow{NH_3}$ (\$\alpha\$-Bromo-\$\delta\$-benzoylamino-\$n\$-valeric acid)

 $\begin{array}{c} \text{C}_{6}\text{H}_{5} \cdot \text{COCl}(\text{NaOH}) \\ \text{C}_{6}\text{H}_{5} \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_{2} \cdot \text{CH}_{2} \cdot \text{CH}(\text{NH}_{2}) \cdot \text{COOH} \\ \hline (\alpha\text{-Amino-}\delta\text{-benzoylamino-}n\text{-valeric acid}) \end{array}$

 $\begin{array}{c} C_6H_5\cdot CO\cdot NH\cdot CH_2\cdot CH_2\cdot CH_2\cdot CH(NH\cdot CO\cdot C_6H_5)\cdot COOH\\ \\ (Ornithuric\ acid) \end{array}$

A synthesis of ornithuric acid from acrolein by the following reactions has been described by Keimatsu and Sugasawa (297, 298):

$$\begin{array}{c} \text{CH}_2\text{:}\operatorname{CH}\cdot\operatorname{CHO} \xrightarrow{\operatorname{HCl}+\operatorname{C}_2\operatorname{H}_5\operatorname{OH}} & \operatorname{ClCH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{OC}_2\operatorname{H}_5)_2 \xrightarrow{\operatorname{HI}+\operatorname{KCN}} \\ & \text{(β-Chloropropionacetal)} \end{array}$$

$$\text{CN} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{OC}_2\text{H}_5)_2 \xrightarrow{\text{(Na+C}_2\text{H}_5\text{OH)}}$$

(β-Cyanopropionacetal)

$$\mathrm{NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(OC_2H_5)_2} \xrightarrow{\mathrm{C_6H_5 \cdot COCl}} \mathrm{(KOH)}$$

 $(\gamma$ -Aminobutyracetal)

 $C_{6}H_{5} \cdot CO \cdot NH \cdot CH_{2} \cdot CH_{2} \cdot CH_{2} \cdot CH(OC_{2}H_{5})_{2} \xrightarrow{H_{2}O(H_{2}SO_{4})}$ $(\gamma - Benzoylaminobutyracetal)$

 $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CHO \xrightarrow{NaHSO_3}$ (γ -Benzoylaminobutyraldehyde)

 $\mathrm{C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(OH)SO_3Na} \xrightarrow{\mathrm{KCN}}$

 $(\gamma$ -Benzoylaminobutyraldehyde acid sulfite)

 $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(OH) \cdot CN \xrightarrow{C_2H_5OH + NH_3}$ (δ -Benzoylaminobutyrocyanohydrin)

 $C_{6}H_{5}\cdot CO\cdot NH\cdot CH_{2}\cdot CH_{2}\cdot CH_{2}\cdot CH(NH_{2})\cdot CN\xrightarrow{C_{6}H_{5}\cdot COCl}\xrightarrow{H_{2}O+C_{5}H_{5}N}$ $(\gamma-Benzoylaminobutyroaminonitrile)$

 $C_6H_5\cdot \mathrm{CO}\cdot \mathrm{NH}\cdot \mathrm{CH}_2\cdot \mathrm{CH}_2\cdot \mathrm{CH}_2\cdot \mathrm{CH}(\mathrm{NH}\cdot \mathrm{CO}\cdot \mathrm{C}_6H_5)\cdot \mathrm{CN}\xrightarrow{\mathbf{H}_2\mathrm{O}(\mathrm{HCl})}$

(γ-Benzoylaminobutyrobenzoylaminonitrile)

 $C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

 $(\alpha$ -Amino- δ -benzoylamino-n-valeric acid)

 $\mathrm{C_6H_5 \cdot COCl} \! + \! \mathrm{H_2O} \! + \! \mathrm{C_5H_5N}$

 $\begin{array}{c} {\rm C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5) \cdot COOH} \\ \\ ({\rm Ornithuric\ acid}) \end{array}$

(3) Aspartic Acid. In 1850, Dessaignes (87) synthesized a few crystals of aspartic acid by heating ammonium acid malate in acid solution. These observations were extended by Wolff (299) in the same year. Aspartic acid diethyl ester was synthesized from acetyl succinic ester by Schmidt and Widmann (300), and aspartic acid from oxaloacetic ester oxime by Piutti (23). Methods for the preparation of aspartic acid in about 90 per cent of the theoretical yield

by the acid hydrolysis of asparagine have been described by Schiff (301), Piutti (302), and Pachlopnik (303).

Keimatsu and Kato (203) synthesized 1.7 gm. of aspartic acid from sodium alcoholate, chloroacetic ester, and aminomalonic ester. While the yield was 55 per cent of the theoretical amount, separation of the desired intermediate, ethane- α -amino- α - α - β -tricarboxylic acid triethyl ester [(C₂H₅O)OC·C(NH₂): [CO(OC₂H₅)]₂], was difficult and could be accomplished only by saponification of the mixture of esters, precipitation of the resulting acids as their silver salts, and fractional crystallization of the final products, the hydrochlorides of iminodiacetic acid and aspartic acid. As shown by Redemann (194), aspartic acid may be synthesized satisfactorily from benzoylaminomalonic ester.

A 4 gm. lot of analytically pure aspartic acid was prepared in approximately 27 per cent of the over-all yield by Dunn and Smart (184) from chloroacetic ester and sodium phthalimidomalonic ester. One of the most satisfactory methods for the synthesis of aspartic acid is that of Dunn and Fox (255). The reactions given below were employed for the preparation of 78 gm. of analytically pure aspartic acid in 59 per cent of the theoretical yield.

(4) Cystine. The syntheses of cystine recorded in the literature are limited to reactions of serine with sulfides. In the synthesis, reported in 1903 by Erlenmeyer (25), and described more fully a

year later by Erlenmeyer and Stoop (304), cystine was prepared by the following reactions:

$$\begin{array}{c} \text{HOCH}_2 \cdot \text{CH(NH} \cdot \text{CO} \cdot \text{C}_6\text{H}_5) \cdot \text{CO(OC}_2\text{H}_5) \xrightarrow{P_2\text{S}_5} \\ \text{(Benzoylserine ethyl ester)} \end{array}$$

$$\begin{array}{c} \text{HCl} \\ \text{HS} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH} \cdot \text{CO} \cdot \text{C}_6\text{H}_5) \cdot \text{CO}(\text{OC}_2\text{H}_5) \longrightarrow \\ \text{(Benzoylcysteine ethyl ester)} \end{array}$$

$$\begin{array}{c} \text{HS} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2 \cdot \text{HCl}) \cdot \text{COOH} \\ \hline + \text{FeCl}_3 + \text{NH}_4 \text{OH} & \text{S} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} \\ & \text{(Cystine)} \end{array}$$

(Cysteine hydrochloride)

In 1908, Fischer and Raske (305) synthesized cystine by a comparable method. l-Serine ester was converted to l- α -amino- β -chloropropionic acid by the action of phosphorous pentachloride. This product was heated with barium hydrosulfide and the resulting cysteine was oxidized to cystine by Erlenmeyer's method. Cystine, partly racemized by exposure to alkali, was obtained in approximately 25 per cent of the theoretical yield.

Neither the Erlenmeyer nor the Fischer synthesis is entirely satisfactory because the beginning substance, serine, cannot be readily obtained and the final inactive product is undoubtedly a mixture of racemic and meso forms. At the present time, dl-cystine is most conveniently prepared by sulfuric acid racemization of l-cystine (279).

(5) Glutamic Acid. Wolff's (29) classical synthesis of glutamic acid was reported in 1890. Equations for these reactions are given below:

$$\begin{array}{c} \operatorname{CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot COOH} + \operatorname{Br_2 \rightarrow CH_3 \cdot CO \cdot C(Br)_2 \cdot CH_2 \cdot COOH} \xrightarrow{H_2O} \operatorname{CO_2} \\ \text{(Levulinic acid)} \qquad \qquad (\beta, \beta\text{-Dibromolevulinic acid)} \end{array}$$

$$+CH_3 \cdot CO \cdot CO \cdot CH_2 \cdot COOH + CH_3 \cdot CO \cdot CO \cdot CH_3 + acrolein-like gas$$
 (Diacetyl carboxylic acid) (Diacetyl)

$$+HCO \cdot CO \cdot CH_2 \cdot CH_2 \cdot COOH$$
 (Glyoxylpropionic acid)

$$\begin{array}{c} \text{HCO} \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} & \text{NH}_2\text{OH} \\ \text{(Glyoxylpropionic acid)} \end{array}$$

HC(:NOH) · C(:NOH) · CH₂ · CH₂ · COOH
$$\longrightarrow$$
 (γ - δ -Diisonitroso- n -valeric acid)

This method has been little used because of difficulties in the isolation and purification of the intermediate substances.

The synthesis of glutamic acid by the reduction of diethyl diazoglutarate has been reported by Chiles and Noyes (306) and Levene and Mikeska (307). A 1.1 gm. lot of glutamic acid in 23 per cent of the theoretical yield was prepared by Knoop and Oesterlin (238) from the hydrogenation and amination of α -ketoglutaric acid in the presence of the catalyst, palladium black. Keimatsu and Sugasawa (108) synthesized glutamic acid from acrolein, but neither the quantity of amino acid prepared nor the percentage yield was stated. Equations for these reactions (see page 63 for the preparation of β -cyanopropionacetal) are given below:

$$\begin{array}{c} \operatorname{CN}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{OC}_2\operatorname{H}_5)_2 \xrightarrow{(\operatorname{C}_2\operatorname{H}_5\operatorname{OH})} \\ \\ (\beta\operatorname{-Cyanopropionacetal}) \\ \\ \operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{OC}_2\operatorname{H}_5)_2 \xrightarrow{\operatorname{H}_2\operatorname{SO}_4} \\ \\ (\gamma\operatorname{-Diethoxy-}n\operatorname{-butyric acid}) \\ \\ \operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CHO} \xrightarrow{\operatorname{KCN}} \operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{OH})\cdot\operatorname{CN} \xrightarrow{\operatorname{NH}_4\operatorname{Cl}} \\ \\ (\operatorname{Suceinic acid} \qquad (\gamma\operatorname{-Hydroxy-}\gamma\operatorname{-eyano-}n\operatorname{-butyric acid}) \\ \\ \operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{NH}_2)\cdot\operatorname{CN} \xrightarrow{\operatorname{H}_2\operatorname{O}} \\ \\ (\gamma\operatorname{-Amino-}\gamma\operatorname{-eyano-}n\operatorname{-butyric acid}) \\ \\ \operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{NH}_2)\cdot\operatorname{COH} \xrightarrow{\operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{NH}_2)\cdot\operatorname{COOH}} \\ \\ \\ \operatorname{HOOC}\cdot\operatorname{CH}_2\cdot\operatorname{CH}_2\cdot\operatorname{CH}(\operatorname{NH}_2)\cdot\operatorname{COOH} \\ \\ \end{array}$$

(Glutamic acid)

Sörensen suggested that glutamic acid could be synthesized from sodium phthalimidomalonic ester and β -chloropropionic ester, but Dunn and Smart (184) were unable to prepare stable condensation products with this or related halogen compounds. However, a satisfactory synthesis of glutamic acid from benzoylaminomalonic ester (see pages 49–50) has been described by Dunn and coworkers (197). A 10 gm. lot of glutamic acid was prepared in a 16 per cent over-all yield calculated from malonic ester.

The most promising synthesis of glutamic acid appears to be the acid hydrolysis of α -pyrrolidone carboxylic acid. The latter compound is prepared by heating dry d-glutamic acid to a high temperature. It is generally assumed that complete dehydration and racemization are effected at temperatures above 200°, although the evidence in the literature on this point is conflicting. In 1882, Haitinger (308) observed that a molecule of water is removed and α -pyrrolidone carboxylic acid (pyroglutamic acid) is formed when glutamic acid is heated for some time at 180 to 190°. From subsequent investigations (264, 265, 309-316, 500), it has been shown that the completeness of dehydration of d-glutamic acid for dealcoholization of glutamic ester (311, 313), as well as the degree to which the resulting α -pyrrolidone carboxylic acid is racemized. vary markedly with the conditions. It has been shown further that equilibrium mixtures of glutamic acid and α-pyrrolidone carboxylic acid in varying stages of racemization are formed when d-glutamic acid, or l-pyrrolidone carboxylic acid, is heated in aqueous, acid, or basic solution. Foreman (265) and Bethke and Steenbock (312) found that long heating periods, elevated temperatures, and high acid or base concentrations hinder the formation of α -pyrrolidone carboxylic acid and favor the reverse change. As may be expected. the transformation of glutamic acid to α -pyrrolidone carboxylic acid in the presence of water is never complete. On the other hand, the hydrolysis of the pyrrolidone ring is almost quantitative when a 5 per cent or stronger solution of acid or base is maintained at the boiling temperature for 24 to 48 hours. This transformation is more rapid with higher concentrations of acid or base.

(6) Glycine. Large quantities of glycine may be prepared conveniently and economically by a combination of the Robertson (123) or the Cheronis (155) method with that of Orten and Hill (125). Chloroacetic acid is allowed to react with concentrated ammonia at room temperatures in a molal ratio of approximately 1:60 or at 60 to 65° with ammonium carbonate in a molal ratio

of 1:4. Excess of ammonia is removed by evaporation, 5 volumes of methyl alcohol are added to the solution, and glycine is allowed to crystallize. Analytically pure glycine may be prepared by washing the crude product with methyl alcohol and recrystallizing it once or twice from methyl alcohol-water solution. The yield of pure amino acid is 55 to 60 per cent of the theoretical amount.

The preparation of glycine from formaldehyde, ammonium chloride, and sodium cyanide (101, 104) or from this aldehyde, ammonia, and anhydrous hydrocyanic acid by the original Strecker method (117) is less convenient and efficient than the chloroacetic acid procedure. The over-all yields by the Strecker methods were only about 26 per cent in the synthesis of 115 to 140 gm. lots of glycine.

Because of the current wide-spread use of glycine in the treatment of certain muscular disorders, there has been renewed interest in the synthesis of this amino acid. Patented processes for this purpose include the hydrolysis of glycine esters with organic acids (317), alkaline hydrolysis of glycine ethyl ester sulfate (318), hydrolysis of methyleneaminoacetonitrile (319), and electrolytic reduction of cyanoformic ester (320).

The following additional syntheses of glycine, at present only of theoretical interest, have been reported: hydrolysis of hippuric acid (321, 322), uric acid (323), hydantoic acid (324), phthalimidoacetic ester (156, 157), thiouramil (325), aminomalonic ester (198), thioglycylglycine thioamide (326), aminoacetonitrile (113), 2thiohydantoin (327), 2, 3, 5, 6-tetraketopiperazine-1-acetic acid (328), and serine (280); reduction of cyanoformic ester (329, 330), isonitrosothioglycollic acid (331), isonitrososulfohydantoin (331), nitromalonamide (188), nitroacetic ester (332), and nitrosomalonic ester (202); reduction and hydrolysis of cyanogen (333) and 4nitroglyoxaline (287); action of ammonium cyanide and sulfuric acid on glyoxal (97) and of ammonium carbonate on glyoxylic acid (241); alkaline cleavage of cholic acid (334); hydrolysis and thermal decomposition of aminomalonic nitrile (187, 335); thermal decomposition of aminomalonic acid (189); reaction of chloroacetic ester with hexamethyleneamine (237, 336) and of chloroacetic acid, ammonia, and cupric hydroxide (126); hydrogenation of hydroxyaminoacetic acid (32); hydrolysis of nitrosohydrazinoacetic ester (337), cyanomethylurethane (338), hydrazinodiacetic acid (339), and of the intermediates formed by the action of nitrous acid on potassium malonhydrazide (205); reduction of diazoacetic ester (204); and decomposition of the azide of phenylcarbamino bis glycylaminoacetic acid (340).

(7) Histidine. Histidine has been synthesized only by Pyman's classical methods. By his first method (36, 159), published in 1911, 4-chloromethylglyoxaline was condensed with ethyl chloromalonate. In the second synthesis (235), glyoxaline formaldehyde was condensed with hippuric acid by Erlenmeyer's azlactone procedure. Equations for these reactions are given below.

Malonic Ester Method

Azlactone Method

Histidine was ioslated as the mono- and di-hydrochloride, the di-picrate, and the free base. A 6.3 gm. quantity of histidine mono-hydrochloride was prepared by the first method, and 3.5 gm. of the di-picrate by the second. The over-all yields, calculated from the diaminoacetone dihydrochloride in the first case, and from hydroxymethylglyoxaline in the second, were approximately 23 and 11 per cent, respectively. Little use has been made of Pyman's syntheses, principally because of the difficulties encountered in some of the steps. Although 79, 50, and 66 per cent yields of crude acetone dicarboxylic acid, diisonitrosoacetone, and the diaminoacetone dihydrochloride-zinc chloride double salt, respectively, were obtained by Pechmann (341), Pechmann and Wehsarg (342), and Kalischer (343), it has not been possible to duplicate these results in the author's laboratory. The preparation of acetone

dicarboxylic acid has been simplified by Jerden (344) by the use of hydrated citric acid and an extra amount of fuming sulfuric acid. However, the product is only about 80 per cent pure due to contamination with ethyl acetoacetate. On the other hand, Gabriel and Posner's (345) synthesis of diaminoacetone dihydrochloride from α - γ -glycerol dichlorohydrin and potassium phthalimide, as modified by J. Corse and L. Katzin in the author's laboratory, gives excellent results.

Gerngross' (346) attempted synthesis of histidine from 4-methylglyoxaline and chloral was unsuccessful. It was expected that the aldehyde would condense with the methyl group, but Windaus (347) showed later that it reacts instead with the 5-carbon atom in the ring. The synthesis of 4-hydroxymethylglyoxaline by a new method was described in 1933 by Jackson and Marvel (348). Although the yield of the final product was not stated, the over-all yield from the first three steps was approximately 7 per cent. Equations for these reactions are given below:

$$\begin{array}{c} CO \\ N \cdot CH_2 \cdot CO \cdot CH_2Br \xrightarrow{CH_3 \cdot COONa} \\ \hline (C_2H_4OH) \\ \hline \\ (\omega\text{-Bromoacetonyl phthalimide}) \\ \hline \\ CO \\ (\omega\text{-Acetoxy acetonyl phthalimide}) \\ \hline \\ (\omega\text{-Acetoxy acetonyl phthalimide}) \\ \hline \\ HCl \cdot NH_2 \cdot CH_2 \cdot CO \cdot CH_2OH \xrightarrow{KCNS} \\ \hline \\ (Hydroxyaminoacetone \\ hydrochloride) \\ \hline \\ CH-NH \\ \hline \\ (2\text{-Thio-4-hydroxymethyl glyoxaline}) \\ \hline \\ CH-NH \\ \hline \\ FeCl_3 \\ \hline \\ CH-NH \\ CH-NH$$

(4-Hydroxymethyl glyoxaline)

(8) β -Hydroxyglutamic Acid. The synthesis of this amino acid was reported by Dakin (39) in 1919. Starting with glutamic acid, β -hydroxyglutamic acid was prepared in 2 per cent over-all yield as a hygroscopic white powder. Owing to the high solubility of this product, a glassy substance, but no crystals, were obtained upon evaporation of its aqueous solution. Equations showing these reactions are given below:

Only traces of impure β -hydroxyglutamic acid could be prepared by reduction of α -isonitrosoacetone dicarboxylic ester [(C₂H₅O) OC·C(:NOH)CO·CH₂·CO(OC₂H₅)], by the reduction of α -isonitroso- β -ethoxyglutaconic ester [(C₂H₅O)OC·C(:NOH)·C(OC₂H₅):CH·CO(OC₂H₅)], or by the following reactions with γ -diethoxyacetoacetic ester:

$$(C_2H_5O)_2CH \cdot CO \cdot CH_2 \cdot CO(OC_2H_5) \xrightarrow{H_2} Na, Hg$$

$$(\gamma\text{-Diethoxyacetoacetic ester})$$

$$(C_2H_5O)_2CH \cdot CH(OH) \cdot CH_2 \cdot CO(OC_2H_5) \xrightarrow{H_2O} H_2SO_4$$

$$(\gamma\text{-Diethoxy-}\beta\text{-hydroxy-}n\text{-butyric acid})$$

$$\begin{array}{c} \text{CHO} \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{COOH} \xrightarrow{\text{HCN}} \\ \text{(Malic semi-aldehyde)} \\ \text{NH}_2 \cdot \text{CH}(\text{CN}) \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{COOH} \xrightarrow{\text{H}_2\text{O}} \\ \text{(β-Hydroxy-γ-amino-γ-cyano-n-butyric acid)} \\ \text{HOOC} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}(\text{OH}) \cdot \text{CH}_2 \cdot \text{COOH} \\ \text{(β-Hydroxyglutamic acid)} \end{array}$$

An attempted synthesis from β -hydroxyglutaric acid [HOOC·CH₂·CH(OH)·CH₂·COOH] through acetyl- β -hydroxyglutaric acid anhydride

$$H_2C$$
 C CH_2 C $CO \cdot CH_3$

and α -bromo- β -acetylhydroxyglutaric acid [HOOC·CH(Br)·CH (OCOCH₃)·CH₂·COOH] failed because ammonia reacts with the α -halogen compound without introducing an amino group. The following additional syntheses were tried without success: reduction of the hydrazone of acetone dicarboxylic ester; condensation of oxymethylene hippuric ester with malonic ester; reduction of o-toluidine azoacetone dicarboxylic ester; condensation of dichloroacetal with malonic ester followed by reactions analogous to Leuch's serine synthesis; reduction of isonitrosoglutaconic ester; reduction of hydroxy isoazole dicarboxylic ester; and reactions with benzoylaminoglutaconic acid.

In 1931, Harington and Randall (38) described an excellent synthesis in which β -hydroxyglutamic acid was produced in approximately 60 per cent over-all yield from acetone dicarboxylic ester. The following reactions were employed:

The preparation of ethyl α -isonitrosoacetone dicarboxylate in almost quantitative yield represented a great improvement over Pechmann's (349) method. In the reduction of this ester with the Raney (nickel) catalyst, Levene and Schormüller (201) isolated two intermediate products and prepared β -hydroxyglutamic acid in somewhat smaller yields than those reported by Harington and Randall. This amino acid contains two asymmetric carbon atoms, and hence the synthetic product may be one or a mixture of epimeric forms.

(9) Hydroxyproline. A synthesis of hydroxyproline was reported by Leuchs (41) in 1905. The series of reactions (shown below) leading to the synthesis of this amino acid was investigated as far as α -bromo- δ -chloro- γ -valerolactone- γ -carboxylic acid ethyl ester by Traube and Lehmann (170) at an earlier date.

A 2 gm. quantity of a difficultly soluble, and a 6.3 gm. of a readily soluble, copper salt of γ -hydroxyproline were isolated by Leuchs.

In a later paper (44), the preparation of 6.9 gm. of this amino acid from α - δ -dichlorovalerolactone in 64 per cent of the theoretical yield is described.

It is evident that the γ -hydroxyproline synthesized by Leuchs is only one of the four compounds which have the empirical formula, $C_5H_9O_3N$, and a hydroxypyrrolidine structure. Of these possible compounds (shown below), either II or III is considered to be the naturally occurring form (see discussion on pages 31 and 32). Since γ -hydroxyproline (compound III) contains two asymmetric carbon atoms, it has four possible optically active and two inactive stereo-isomeric modifications. Leuchs separated the latter by fractional crystallization of their copper salts.

Isomeric Hydroxyprolines

These isomeric forms were also isolated by Hammarsten (350) who synthesized γ -hydroxyproline by the following reactions:

$$\begin{array}{c} \operatorname{CH}_2\colon \operatorname{CH} \cdot \operatorname{CH}_2 \cdot \operatorname{CH}(\operatorname{NH} \cdot \operatorname{CO} \cdot \operatorname{C}_6 \operatorname{H}_5) \cdot \operatorname{CO}(\operatorname{OC}_2 \operatorname{H}_5) & \operatorname{Br}_2(\operatorname{HCCl}_3) \\ & (\operatorname{Allyl \ hippuric \ ester}) & \\ \operatorname{CH}_2(\operatorname{Br}) \cdot \operatorname{CH}(\operatorname{Br}) \cdot \operatorname{CH}_2 \cdot \operatorname{CH}(\operatorname{NH} \cdot \operatorname{CO} \cdot \operatorname{C}_6 \operatorname{H}_5) \cdot \operatorname{CO}(\operatorname{OC}_2 \operatorname{H}_5) & \\ & (\operatorname{Ethyl} - \gamma - \delta \text{-dibromo-} \alpha \text{-benzoylamino-} n \text{-valeric acid}) & \operatorname{Ba}(\operatorname{OH})_2 \\ & \operatorname{HOCH}_2 \cdot \operatorname{CH} - \operatorname{CH} \cdot \operatorname{NH} \cdot \operatorname{CO} \cdot \operatorname{C}_6 \operatorname{H}_5 + \operatorname{CH}_2 - \operatorname{CH} \cdot \operatorname{O} \cdot \operatorname{CO} \cdot \operatorname{C}_6 \operatorname{H}_5 \\ & \circ - \operatorname{CO} & \operatorname{HOOC} \cdot \operatorname{CH} & \operatorname{CH}_2 & \\ & \operatorname{NH} \\ & (\alpha \text{-Benzoylamino-} \delta \text{-hydroxy-} & [\gamma \text{-}(\operatorname{Benzoylhydroxy}) \operatorname{proline}] \end{array}$$

(α-Benzoylamino-δ-hydroxy- [γ -(Benzoylhydroxy)proline] valerolactone)

- (10) Iodogorgoic Acid. This amino acid is synthesized from tyrosine by the methods described by Wheeler and Jamieson (47), Henze (48), Oswald (351), Abderhalden (352), and Harington (73). Tyrosine, dissolved in sodium or ammonium hydroxide, is treated with powdered iodine or a solution of iodine in potassium iodide. The solution is evaporated and the product is recrystallized from water or 50 per cent acetic acid. Yields varying from 48 to almost 100 per cent of the theoretical were reported by these authors. In the author's laboratory, duplication of these results and the preparation of an analytically pure product, have not been accomplished.
- (11) **Isoleucine.** Isoleucine was first synthesized in 1905 by Bouveault and Locquin (243, 355, 356). Secondary butyl iodide, prepared by Romburgh's (161) method, was allowed to react with acetoacetic ester as shown by the following equations:

$$\begin{array}{ll} \mathrm{CH_3 \cdot CH_2 \cdot CH(CH_3)I + CH_3 \cdot CO \cdot CH_2 \cdot CO(OC_2H_5) + NaOC_2H_5 \rightarrow} \\ & (Sec. \ butyl \ iodide) & (Acetoacetic \ ester) \\ \mathrm{CH_3 \cdot CO \cdot CH} \left[\mathrm{CH(CH_3)(CH_2 \cdot CH_3)} \, \right] \mathrm{CO(OC_2H_5)} \xrightarrow[\mathrm{sulfate}]{\mathrm{nitrosyl}} \\ & \xrightarrow[\mathrm{sulfate}]{\mathrm{nitrosyl}} \end{array}$$

(Sec. butylacetoacetic ester)

$$\mathrm{CH_3} \cdot \mathrm{CH_2} \cdot \mathrm{CH}(\mathrm{CH_3}) \cdot \mathrm{C}(:\mathrm{NOH}) \cdot \mathrm{COOH} \xrightarrow{\mathrm{H_2} + \mathrm{HCl} + \mathrm{C_2H_5OH}}$$

 $(\beta$ -Methyl- β -methyl pyruvic oxime)

$$\mathrm{CH_3} \cdot \mathrm{CH_2} \cdot \mathrm{CH}(\mathrm{CH_3}) \cdot \mathrm{CH}(\mathrm{NH_2})$$
 $\mathrm{CO}(\mathrm{OC_2H_5})$

(Isoleucine ethyl ester)

With zinc and hydrochloric acid, sodium amalgam, and aluminum amalgam as reducing agents, the yields of isoleucine ester were 60 to 70, 35, and 25 per cent of the theoretical, respectively.

In 1907, Ehrlich (50) synthesized isoleucine from d-sec. valeraldehyde, ammonia, and hydrocyanic acid. Only 7.5 gm. of isoleucine, isolated as the copper salt, were prepared, and the yield of aldehyde was only 25 per cent of the theoretical amount. Since the product

could be separated into three fractions of markedly different specific rotations, it was assumed to be a mixture of d-isoleucine and d-alloisoleucine. It may be noted that the presence of two asymmetric carbon atoms in isoleucine permits the existence of four active and two inactive stereochemical forms.

A synthesis of isoleucine by the malonic ester method was reported on April 4,1908 by Brasch and Friedmann (162), and twenty-three days later by Ehrlich (51). Approximately 20 gm. of isoleucine in an over-all yield of about 53 per cent, calculated from α -bromo-sec. butylmalonic acid, were prepared by the former workers, while Ehrlich synthesized 5 gm. of isoleucine in an over-all yield of approximately 18 per cent calculated from malonic ester. A 35 to 45 per cent over-all yield of isoleucine was prepared by Abderhalden and Zeisset (53) who used the malonic ester method. The relatively low yields of isoleucine are caused by the formation of oily side reaction products of unknown constitution.

A convenient synthesis of isoleucine from sec. butyl carbinol through the corresponding bromide and nitrile is described on page 44.

(12) Leucine. Leucine was first synthesized from isovaleraldehyde, hydrocyanic acid, and ammonia by Limpricht (54) in 1855. The yield was not given by him or by subsequent investigators (1, 89, 107). Ehrlich (50) prepared 65 gm. of this amino acid in approximately 48 per cent of the theoretical yield. Leucine has also been synthesized from isovaleraldehyde and hydantoin by Pinner and Spilker (207), from isobutylaldehyde and hippuric acid by Erlenmeyer and Kunlin (232), and from isobutyl iodide and acetoacetic ester by Bouveault and Locquin (357). However, these authors make no statements of quantities or yields.

A practicable synthesis of leucine from malonic ester and isobutyl bromide was described in 1906 by Fischer and Schmitz (163). Although these authors carried these reactions only to the preparation of α -bromoisocaproic acid, the over-all yield calculated from these results and those obtained earlier by Fischer (132) from the amination of the bromo-acid, was approximately 37 per cent of the theoretical amount.

A satisfactory synthesis of leucine from isoamyl alcohol through the corresponding bromide and nitrile is described on page 44.

(13) Lysine. Lysine was first synthesized by Fischer and Weigert (56) who prepared 5.3 gm. of this amino acid (as the picrate) in a 15.5 per cent over-all yield from malonic ester and γ -chloro-

butyronitrile. The equations for these reactions are given below:

$$\begin{array}{c|cccc} CO(OC_2H_5) & & + NaOC_2H_5 + ClCH_2 \cdot CH_2 \cdot CH_2 \cdot CN \rightarrow \\ & & + CO(OC_2H_5) & & & + CO(OC_2H_5) & & + CO($$

The γ -chlorobutyronitrile required for this synthesis may be readily prepared by the method of Henry (358). The principal objection to Fischer and Weigert's method seems to be the low yield (32 per cent of the theoretical yield) obtained in the final reduction step. Difficulties were encountered by these authors in their attempts to prepare lysine from potassium phthalimide and tetramethylene bromide by the method employed for the synthesis of ornithine (289). Likewise, Sörensen's synthesis of lysine from phthalimido- δ -phthalimidotetramethylenemalonic ester (see discussion on pages 47–49) was not entirely satisfactory.

In 1909, von Braun (359) described a synthesis of lysine from benzoylpiperidine by standard reactions in all except the first step. Equations for these reactions are given below:

$$\begin{array}{c} CH_2-CH_2\\ C_6H_5\cdot CO\cdot N \\ CH_2-CH_2\\ (Benzoylpiperidine) \\ C_6H_5\cdot CO\cdot NH\cdot (CH_2)_4\cdot CH_2Cl-\\ \\ CH_2-CH_2\\ (Benzoyl-\epsilon\text{-chloroamyl amine}) \\ \\ C_6H_5\cdot CO\cdot NH\cdot (CH_2)_4\cdot CH_2\cdot CN-\\ \hline \\ (Benzoyl-\epsilon\text{-cyanoamylamine}) \\ \end{array}$$

$$\begin{array}{c} {\rm C_6H_5\cdot CO\cdot NH\cdot (CH_2)_4\cdot CH_2\cdot COOH} \xrightarrow{Br_2} \\ \\ {\rm (ϵ-Benzoylamino-n-caproic acid)} \\ {\rm C_6H_5\cdot CO\cdot NH\cdot (CH_2)_4\cdot CH (Br)\cdot COOH} \xrightarrow{NH_3} \\ \\ {\rm (α-Bromo-ϵ-benzoylamino-n-caproic acid)} \\ {\rm C_6H_5\cdot CO\cdot NH\cdot (CH_2)_4\cdot CH (NH_2)\cdot COOH} \xrightarrow{H_2O} \\ \\ {\rm (α-Amino-ϵ-benzoylamino-n-caproic acid)} \\ {\rm (α-Amino-ϵ-benzoylamino-n-caproic acid)} \\ {\rm HCl\cdot NH_2\cdot (CH_2)_4\cdot CH (NH_2\cdot HCl)\cdot COOH} \\ \\ {\rm ($Lysine dihydrochloride)} \end{array}$$

In more recent investigations, Marvel and co-workers (360) found that the yield of ϵ -benzoylamino-n-caproic acid can be increased by complete hydrolysis of benzoylamino- ϵ -cyanoamine to ϵ -amino-n-caproic acid and subsequent re-benzoylation of the latter compound. Other improvements in this synthesis were proposed by these authors, although no statement was made of the yield of amino acid.

A new synthesis of lysine from acrolein was described in 1927 by Sugasawa (298). γ -Benzoylamino-n-butyraldehyde, prepared by the reactions indicated on page 63, was converted to ϵ -benzoylamino-n-caproic acid by the reactions shown below. Lysine was prepared from the latter compound by von Braun's method.

$$\begin{array}{c} C_{6}H_{5}\cdot CO\cdot NH\cdot CH_{2}\cdot CH_{2}\cdot CH_{2}\cdot CHO & \xrightarrow{C_{5}H_{5}N} \\ \hline C_{5}H_{11}N+NH_{2}(COOH)_{2} \end{array} \\ (\gamma\text{-Benzoylamino-}n\text{-butyraldehyde}) \\ C_{6}H_{5}\cdot CO\cdot NH\cdot (CH_{2})_{3}\cdot CH: CH\cdot COOH & \xrightarrow{\text{Catalytic}} \\ \hline \text{reduction} \\ [\beta\text{-}(\gamma\text{-Benzoylaminopropyl}) \text{ acrylic acid}] \\ C_{6}H_{5}\cdot CO\cdot NH\cdot (CH_{2})_{4}\cdot CH_{2}\cdot COOH & \xrightarrow{\text{von Braun's}} \\ \hline \text{tysine} \\ (\epsilon\text{-Benzoylamino-}n\text{-caproic acid}) \end{array}$$

However, these reactions appear to be less advantageous than those proposed originally by von Braun.

At the present time, lysine is synthesized most satisfactorily from commercial cyclohexanone by the method of Eck and Marvel (361). These authors prepared 75 gm. of lysine dihydrochloride in

23 per cent over-all yield calculated from cyclohexanone. Equations for these reactions are given below:

$$\begin{array}{c} CH_2 \\ CH_2 \\ CO \\ \hline \\ CH_2 \\ CO \\ \hline \\ CH_2 \\ CH_2$$

The preparation of cyclohexanone oxime by the indirect method of Semon (362), and the rearrangement of this oxime in 10 gm. lots by Ruzicka's (363) modification of Wallach's (364) method, are points of particular interest in this series of reactions. This excellent method has been used in the author's laboratory for the preparation of large quantities of lysine dihydrochloride.

(14) **Methionine.** Methionine was first synthesized in 1928 by Barger and Coyne (59). β -Methylthiolpropionaldehyde (CH₃·S·CH₂·CH₂·CHO), prepared from methylmercaptan (CH₃SH) and β -chloropropionaldehyde acetal [ClCH₂·CH₂·CH(OC₂H₅)₂] through β -methylthiolpropionaldehyde acetal [CH₃·S·CH₂·CH₂·CH₂·CH(OC₂H₅)₂], was subjected to the modified Strecker reaction. Although the quantity of amino acid prepared was not stated, the over-all yield was 3.4 per cent of the theoretical amount. Despite efforts to improve the isolation of methionine, the yield calculated from the aldehyde was only 6 per cent of the theoretical yield.

Barger and Coyne's attempts to synthesize methionine by the hydantoin method were unsuccessful. Although methylthiolethylidenehydantoin was prepared in an approximately 10 per cent over-

$$CH_3 \cdot S \cdot CH_2 \cdot CH = C$$

$$CO - NH$$

all yield from methyl mercaptan and bromoacetal through the intermediate methylthiolacetal $[CH_3 \cdot S \cdot CH_2 \cdot CH(OC_2H_5)_2]$, the reduction of the hydantoin derivative gave only decomposition products and an uncrystallizable syrup.

A malonic ester synthesis of methionine was reported by Windus and Marvel (167) in 1930. Methyl isothiourea sulfate, prepared in 90 to 95 per cent yields by the method of Arndt (365), was hydrolyzed with sodium hydroxide. The liberated methyl mercaptan was absorbed in alcoholic sodium ethylate solution. The further reactions in this synthesis are shown below:

Only about 2 gm. of methionine were prepared and the over-all yield, calculated from methyl mercaptan, was only about 2 per cent of the theoretical amount. The yield of methionine, calculated from β -methylthiolethyl ethylmalonic acid, was only 24 per cent of the theoretical amount because of difficulties in the bromination, decomposition, and amination of this intermediate. However, this synthesis has been improved by Schmidt and co-workers (168). The yield of β -methylthiolethyl malonic ester was increased from 45 to 67 per cent of the theoretical by using an excess of malonic ester, and the yield of methionine was increased by decarboxylating the hydrobromide of β -methylthiolethyl aminomalonic acid in xylene. By adoption of the xylene method, Marvel (366) prepared methionine in 45 to 50 gm. lots in an over-all yield of slightly less than 4 per cent of the theoretical amount. These results have been confirmed by Stoddard in the author's laboratory.

In 1934, Barger and Weichselbaum (183) adapted the phthalimidomalonic ester synthesis to the preparation of approximately 8 gm. of methionine in 54 to 60 per cent over-all yields calculated from β -methylthiolethyl chloride. On this basis, the over-all yields by the method of Windus and Marvel, and the modified procedure of Marvel, were approximately 7 and 15 per cent, respectively. Some difficulties have been experienced with the phthalimidomalonic ester synthesis in the author's laboratory. A new synthesis

of methionine from α -benzamido- γ -butyrolactone has recently been described by Hill and Robson (498).

(15) Norleucine. The synthesis of norleucine from (a) α -bromon-caproic acid and ammonia and (b) valeraldehyde, ammonia, and hydrocyanic acid was reported by Hüfner (109) in 1870. However, it seems to be uncertain whether the aldehyde used by Hüfner was the normal or the iso compound (1, 367, 368).

Large quantities of norleucine may be conveniently synthesized by the reactions which are given on page 41. It has been shown that norleucine may be prepared from ammonia and α -bromo-n-caproic acid in approximately 65 per cent of the theoretical yield (137–140). n-Caproic acid may be obtained from commercial sources, it may be synthesized from commercial n-amyl chloride by the reactions given on page 44, or it may be prepared from n-butyl bromide (or iodide) and malonic ester in approximately 66 per cent of the theoretical yield (139, 164, 169). α -Bromo-n-caproic acid may be synthesized from n-butylmalonic ester (139) in 75 per cent, or from n-caproic acid (137, 367) in 81 to 89 per cent of the theoretical yield.

(16) Phenylalanine. The synthesis of phenylalanine from cinnamic acid through the intermediate α -chloro- β -phenylpropionic acid was considered by Erlenmeyer (369) in 1879. However, this method was abandoned when it was found that either β -, or a mixture of α - and β -, halogen compounds are formed from cinnamic and hydrochloric or hypochlorous acids.

Phenylalanine was first synthesized from phenylacetaldehyde, ammonia, and hydrocyanic acid by Erlenmeyer and Lipp (68, 110). A 48 gm. lot of crude phenylalanine was prepared in a 74 per cent over-all yield calculated from the intermediate phenyl- α -hydroxy-propionitrile.

The synthesis of phenylalanine from benzaldehyde and hippuric acid was first investigated by Plöchl (220, 221). An extensive study of the factors governing these reactions and the constitution of the intermediate azlactones was made by Erlenmeyer and Kunlin (222–228). Later workers (229, 230) improved this synthesis by introducing hydriodic acid and red phosphorous for the simultaneous reduction and hydrolysis of the intermediate α -benzoylaminoacrylic acid. By taking advantage of these improvements, Gillespie and Snyder (231) prepared 10.5 to 11 gm. lots of phenylalanine in approximately 40 per cent of the over-all theoretical yield.

From 7 to 30 gm. of phenylalanine have been synthesized in approximately 50 to 60 per cent over-all yields by use of the following reactions: (a) sodium phthalimidomalonic ester and benzyl chloride (177, 178), (b) malonic ester and benzyl chloride (171), (c) hydantoin and benzaldehyde (209), and (d) diketopiperazine and benzaldehyde (216). Phenylalanine may be synthesized satisfactorily by any of the foregoing methods. Phenylalanine may also be prepared by the reduction of the α -oxime of β -phenylpropionic acid (224, 244–247), reduction and amination of phenylpyruvic acid (238, 239), condensation of benzaldehyde with 2-thio-3-benzoylhydantoin (213), reaction between potassium benzylmalonate and hydrazine (205), and reaction between aminomalonic ester and benzyl chloride (196, 198). Difficulties in the last method may be eliminated by the use of benzoylaminomalonic ester (194, 197).

(17) Proline. Proline was first synthesized in 1900 by Willstätter (69). It was expected that ornithine would be formed by these reactions (shown below), but the synthesis led unexpectedly to proline owing to the instability of the intermediate diamino compound. In a later investigation, Willstätter and Ettlinger (174)

$$\begin{array}{c|cccc} CO(OC_2H_5) & CO(OC_2H_5) \\ & & Br_2 & C(Br)CH_2 \cdot CH_2 \cdot CH_2Br \\ \hline & CO(OC_2H_5) & CO(OC_2H_5) \\ \hline & (\gamma\text{-Bromopropyl} & (\gamma\text{-Bromopropyl-bromomalonic ester}) \\ \hline & CO(OC_2H_5) & CH_2 - CH_2 \\ \hline & C(NH_2) \cdot CH_2 \cdot CH_2 \cdot CH_2NH_2 \\ \hline & CO(OC_2H_5) & CH_2 - CH_2 \cdot CH_2 \cdot CH_2NH_2 \\ \hline & CO(OC_2H_5) & NH \\ & (\gamma\text{-Aminopropyl-aminomalonic ester}) & NH \\ \hline & (\gamma\text{-Aminopropyl-aminomalonic ester}) & CH_2 - CH_2 \\ \hline & Ag_2O & Ag_2O & Ag_2O \\ \hline & (HCl) & NH \cdot HCl & NH \\ \hline & (Proline hydro-chloride) & CProline—isolated as copper salt) \\ \hline \end{array}$$

prepared α , α -pyrrolidine carbonic acid diamide in 12 per cent overall yield calculated from malonic ester, but the yield of proline was not stated. With excess malonic ester, Leuchs (172) increased the yield of γ -bromopropylmalonic ester from 26 to 28.5 per cent to about 38 per cent of the theoretical yield.

In 1901, Fischer (292) synthesized a small amount of proline by means of the following reactions:

Seven years later, a modification of this synthesis was described by Sörensen and Andersen (179). By the reactions shown below, a 22.9 gm. lot of copper prolinate was prepared in approximately 70 per cent of the theoretical over-all yield calculated from sodium phthalimidomalonic ester. The intermediate γ -bromopropylphthalimidomalonic ester was prepared as an uncrystallizable oil of only about 80 per cent purity. This synthesis of proline is thought to be one of the best.

The synthesis of proline from piperidine was reported by Fischer and Zemplén (296) in 1909. α -Bromo- δ -benzoylamino-n-valeric acid, prepared by the reactions shown on page 63, was converted to proline hydrochloride by acid hydrolysis. A 2.5 gm. lot of copper prolinate was synthesized in approximately 34 per cent over-all yield calculated from δ -benzoylamino-n-valeric acid. This method is somewhat unsatisfactory because the yield of the latter compound is low and the bromo derivative is readily attainable only as an impure oil. While the bromo compound is easily separated from the contaminating β , β -dibromo- α -piperidone (given below) by extracting

the oil with sodium carbonate solution, apparently other side reaction products are not removed by this treatment. In the same year, Fischer and Zemplén (370) described an improved technique for the oxidation of arylpiperidine derivatives. Benzoylamino-n-valeric and m-nitrobenzoylamino-n-valeric acids were obtained in 40 to 50 per cent and 60 per cent yields, respectively, by the oxidation of benzoyl and m-nitrobenzoylpiperidine. Proline was synthesized by means of the following reactions:

$$\begin{array}{c} \text{CH}_2\text{---}\text{CH}_2\\ \text{CH}_2 & \text{N}\cdot\text{CO}\cdot\text{C}_6\text{H}_4\cdot\text{NO}_2 \\ \text{----}\text{CH}_2\text{---}\text{CH}_2\\ (\textit{m-Nitrobenzoylpiperidine}) \end{array}$$

HOOC ·
$$\operatorname{CH}_2$$
 · CH_2 · CH_2 · NH · CO · $\operatorname{C}_6\operatorname{H}_4$ · NO_2 by HCl [δ -(m -Nitrobenzovlamino)- n -valeric acid]

A 30 gm. lot of the dl-m-nitrobenzoylproline was prepared in approximately 16 per cent of the theoretical yield calculated from m-nitrobenzoylpiperidine. However, this product was resolved into its active components prior to the preparation of 1.6 gm. of d-copper prolinate.

Heymons' (371) synthesis of proline from α -piperidone through the intermediate β , β -dichloro- α -piperidone is of interest in connection with the previously described methods of Fischer and Zemplén. An 8 gm. quantity of copper prolinate was prepared in approximately 28 per cent over-all yield, calculated from α -piperidone, by the following reactions:

$$\begin{array}{c|c} CH_2 & CH_2 & CH_2 \\ CH_2 & CH_2 & PCl_5 \\ CH_2 & CO & (xylene) \\ \hline \\ NH & (\alpha\text{-Piperi-done}) \\ \hline \\ NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot C(Cl)_2 \cdot COOH \\ \hline \\ NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot C(Cl)_2 \cdot COOH \\ \hline \\ NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot C(Cl)_2 \cdot COOH \\ \hline \\ (Na, Hg) \\ \hline \\ NH \\ (\alpha, \alpha\text{-Dichloro-δ-amino-n-valeric acid)} \\ \hline \\ (Proline) \\ \hline \end{array}$$

The reduction of α -pyrrolidone carboxylic ester to proline was reported by Fischer and Boehner (313) in 1911. Because this ester may be readily prepared from dehydrated d-glutamic acid (see page 68 for a discussion of this topic), this synthesis of proline would be one of the best if it were not for the inherent difficulties. Fischer and Boehner were able to prepare the ester in only 41 per cent of the theoretical yield. Furthermore, the reduction of the ester with sodium and alcohol was not complete and only about 0.8 gm. of proline, isolated as the copper salt, was produced.

McCay and Schmidt (372) found that the final product is grossly impure as shown by the Van Slyke amino nitrogen analysis. It is not improbable that these results may be due to glutamic acid which escaped de-hydration or was formed by hydrolysis of the pyrrolidone (and possibly the pyrrolidine) ring during the treatment with nitrous acid. According to these authors, crude proline may be entirely freed from amino nitrogen by repeatedly crystallizing the copper salt or the salt of phosphotungstic acid. McCay and Schmidt (266) were unsuccessful in their attempts to prepare the oxime or phenylhydrazone of α -pyrrolidone carboxylic acid, to reduce α -pyrrolidone carboxylic acid with eight different catalytic agents, and to reduce the ester with a platinum catalyst.

The efforts of several investigators (266, 373, 374) to prepare proline by the reduction of α -pyrrolcarbonic acid or ester likewise failed. However, in 1930 Putochin (375) obtained 23 per cent of the theoretical yield of copper prolinate by repeated treatment of 1 gm. of 2-carbethoxypyrrole in alcoholic hydrochloric acid solution with electrolytic hydrogen, ferric chloride, and relatively large amounts of platinum oxide. Decided improvements in the reduction of pyrrole derivatives have been made recently by Signaigo and Adkins (376) who prepared 5.2 gm. of proline in 31.8 per cent over-all yield by use of the following reactions:

Under the described experimental conditions, reduction was complete in 1 hour, and the yield of 1,2-dicarbethoxypyrrolidine was 98 per cent of the theoretical amount. Five hours were required for the acid hydrolysis of the latter compound. While the advantages of this direct reduction procedure are apparent, its adaptation to the preparation of larger quantities of proline may be difficult.

The synthesis of proline from aminomalonic ester and trimethylene bromide was described by Putochin (192) in 1923. While this method has inherent limitations, it seems probable that a satisfactory synthesis could be devised from benzoylaminomalonic ester (see pages 49 to 50 for a discussion of these reactions).

A synthesis of proline analogous to that of lysine (361) was investigated by Schniepp and Marvel (377). Although ε-benzoylamino-n-caproic acid, an intermediate in the preparation of lysine from cyclohexanone, could be readily crystallized, the homologous δ-benzoylamino-n-valeric acid could be prepared only as an impure oil. Furthermore, a considerable quantity of the alkali insoluble N-benzoyl- β , β -dibromo- α -piperidone was formed as a side reaction product. α -Piperidone and δ -amino-n-valeric acid were obtained in 60 and 80 per cent of the theoretical yields, respectively. Fischer and Zemplén (296) also failed in their attempts to crystallize δ-benzoylamino-n-valeric acid, prepared from piperidine. However, the homologous m-nitrobenzovlamino-n-valeric acid was readily obtained in crystalline form by these authors (370). By taking advantage of this discovery, a practicable synthesis of proline has been developed by J. Corse and J. Hillis in the author's laboratory. Equations for these reactions are given below:

$$\begin{array}{c} \operatorname{CH_2} & \operatorname{NaNO_2} & \operatorname{CH_2} & \operatorname{H_2SO_4} \\ \operatorname{CH_2} & \operatorname{CH_2} & \operatorname{CH_2} & \operatorname{CH_2} & \operatorname{CH_2} \\ \operatorname{CH_2-CO} & \operatorname{CH_2} & \operatorname{CH_2} & \operatorname{CH_2} & \operatorname{CH_2} \\ \operatorname{CH_2-C} & \operatorname{NoH} & \operatorname{CH_2-CH_2-CH_2-CO} \\ \end{array}$$

$$(\operatorname{Cyclopenta-none}) & \operatorname{(Cyclopenta-none oxime)} & \operatorname{(C2-Keto-penta-methylene imine)} \\ \operatorname{NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH} & + & & & \operatorname{NO_2 \cdot C_6H_4 \cdot COCl} \\ \operatorname{(\delta-Amino-}n\text{-}valeric acid)} & & \operatorname{(}n\text{-}\operatorname{Nitrobenzoyl chloride)} \\ \operatorname{NO_2 \cdot C_6H_4 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH} & \xrightarrow{\operatorname{Br_2}} \\ \operatorname{NO_2 \cdot C_6H_4 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot COOH} & \xrightarrow{\operatorname{Br_2}} \\ \end{array}$$

 $[\delta$ -(m-Nitrobenzoylamino)-n-valeric acid]

$$NO_2C_6H_4 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(Br) \cdot COOH$$

 $[\delta-(m-Nitrobenzoylamino)-\alpha-bromo-n-valeric acid]$

(18) Serine. A seemingly plausible, direct synthesis of serine from ammonia and α -chloro- β -hydroxypropionic acid was investigated at an early date by Melikoff (378) and by Erlenmeyer (379). However, it was found that this reaction led to the formation of isoserine (α -hydroxy- β -aminopropionic acid) instead of serine (α -amino- β -hydroxypropionic acid). Unfortunately, a mixture of α -chloro- β -hydroxy- and α -hydroxy- β -chloropropionic acids results from the reaction between hypochlorous and acrylic acids. Furthermore, the α -chloro- β -hydroxy compound was shown to react with ammonia through the intermediate oxyacrylic acid (glycidsäure) (CH₂—CH COOH) to give isoserine. The synthesis of this un-

natural amino acid from α -halogen- β -hydroxypropionic acid has been described by Fischer and Leuchs (71) and by Neuberg and Mayer (380).

Likewise, Erlenmeyer's (381) attempted synthesis of serine from glycine and formaldehyde failed. This result was unexpected in view of his success in preparing phenylserine [C₆H₅·CH(OH)·CH-(NH₂)·COOH] from benzaldehyde and glycine (381, 382).

Serine was first synthesized by Erlenmeyer (383) in 1902. Equations for these reactions are given below:

$$\begin{array}{ll} HCO(OC_2H_5) + NaOC_2H_5 + C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CO(OC_2H_5) \longrightarrow \\ \\ (Formic \ ester) & (Hippuric \ ester) \\ C_6H_5 \cdot CO \cdot NH \cdot C(:CHONa) \cdot CO(OC_2H_5) & \underbrace{HCl} \\ \\ (Sodium \ salt \ of \ hydroxymethylene \ hippuric \ ester) \\ C_6H_5 \cdot CO \cdot NH \cdot C(:CHOH) \cdot CO(OC_2H_5) & \underbrace{H_2} \\ \\ (Na, Hg) \\ \\ (Hydroxymethylene \ hippuric \ ester) \end{array}$$

$$\begin{split} \mathrm{C_6H_5 \cdot CO \cdot NH \cdot CH(CH_2OH) \cdot CO(OC_2H_5)} &\xrightarrow{H_2O} \\ & (\mathrm{Benzoylserine\ ethyl\ ester}) \\ & \mathrm{HOCH_2 \cdot CH(NH_2) \cdot COOH} \\ & (\mathrm{Serine}) \end{split}$$

Two years later, Erlenmeyer and Stoop (304) presented a more detailed account of this synthesis. Although the quantity of serine which was prepared was not given, the over-all yield, calculated from formic ester, was approximately 48 per cent of the theoretical amount.

Serine was synthesized from glycolaldehyde, hydrocyanic acid, and ammonia by Fischer and Leuchs (71) in 1902. In this synthesis, α -amino- β -(and γ -) hydroxyacids were prepared for the first time by the Strecker method. However, only 2 gm. of serine in 9 per cent of the theoretical yield were prepared. Except for its theoretical interest, this method is of little value since glycolaldehyde polymerizes readily and is easily obtainable only in dilute aqueous solution.

An improved synthesis from the more stable ethoxyacetaldehyde was reported by Leuchs and Geiger (105) in 1906. These reactions are given below:

Because ethoxyacetaldehyde cannot be isolated readily in the pure state, its approximately 46 per cent aqueous solution was utilized. Serine was prepared in 22.6 to 25.8 gm. lots and approximately 14 per cent of the over-all theoretical yield, calculated from chloroacetal, was obtained. These results have been confirmed by other investigators who prepared serine in quantities as large as 340 gm. by this method (201, 305, 384, 385). The preparation of the required intermediate substances, given in the following list, has been described by other workers: ethoxyacetaldehyde from ethoxy-

acetal (386, 387), ethoxyacetal from chloroacetal (388) and bromoacetal (201, 389), chloroacetal from dichloroether (305, 388, 390) or ethyl alcohol (391), bromoacetal from glycolacetal (389) or paraldehyde (201, 392), α,β -dichlorodiethyl ether from diethyl ether (388, 390), and α - β -dibromodiethyl ether from α -chlorodiethyl ether (393). The preparation of ethoxyacetaldehyde by oxidation of the ethyl ether of ethylene glycol, now an inexpensive commercial product, was reported in 1934 by Dunn and coworkers (106). Using a 46 per cent aqueous solution of the aldehyde. these authors prepared 36 gm. of analytically pure serine in 40 per cent of the theoretical yield.

In 1930, Mitra (182) described a satisfactory synthesis of serine from sodium phthalimidomalonic ester and monochlorodimethyl ether. Although less than 2 gm. lots of serine were prepared, the over-all yields, calculated from sodium phthalimidomalonic ester, were 13.5 to 27.7 per cent of the theoretical. Schiltz and Carter (501) have recently described a new synthesis of serine. They use methyl acrylate as starting material. The reactions are:

$$\begin{array}{l} \mathrm{CH_2:CH\cdot CO(OCH_3)\to CH_2(OCH_3)\cdot CH(HgOAe)\cdot CO(OCH_3)\to} \\ \mathrm{(Methyl\ acrylate)} \\ \mathrm{CH_2(OCH_3)\cdot CH(HgBr)\cdot CO(OCH_3)\to CH_2(OCH_3)\cdot CH(Br)\cdot COO\cdot CH_3\to} \\ \mathrm{CH_2(OCH_3)\cdot CH(Br)\cdot COOH\to CH_2(OCH_3)\cdot CH(NH_2)\cdot COOH\to} \\ \mathrm{HO\cdot CH_2\cdot CH(NH_2)\cdot COOH} \\ \mathrm{(Serine)} \end{array}$$

From 150 gm. of methyl acrylate they obtained 12 to 15 gm. of serine.

(19) Thyroxine. Harington and Barger's (72) classical synthesis of thyroxine was reported in 1927. A 0.4 gm. lot of this amino acid was prepared by the reactions indicated below. Although the yields were not given in three of the steps, in the others they were 67, 70 to 100, almost 100, 25, and 50 per cent of the theoretical.

$$(CH_3O)C_6H_4OH + I \longrightarrow NO_2 + K_2CO_3 \ in \ CH_3 \cdot CO \cdot C_2H_5 \rightarrow \\ (Quinol\ mono- \ (3:4:5-Triiodonitro-$$

methyl ether)

benzene)

 α -Amino- β -{3:5-diiodo-4-(4'-hydroxy-phenoxy) phenyl} propionic acid

HO
$$I$$
 $CH_2 \cdot CH(NH_2) \cdot COOH$ I $(Thyroxine)$

By the simultaneous reduction and hydrolysis of the azlactone with hydriodic acid, red phosphorous, and acetic anhydride, Harington and McCartney (229) increased the yield of α -amino- β - (3:5-diiodo-4-(4'-hydroxyphenoxy)phenyl) propionic acid from 25 to 82 per cent of the theoretical.

An improved synthesis of thyroxine from hydroquinone monomethyl ether and 3,4,5-triiodonitrobenzene has been recently described by Savitzkiĭ (497).

(20) Tryptophane. Ellinger and Flamand (77) synthesized this amino acid from indole aldehyde and hippuric acid by Erlenmeyer's azlactone method. Approximately 0.5 gm. of tryptophane was prepared in 10 to 17 per cent over-all yield. The condensation reaction was later investigated by Restelli (394). A synthesis of tryptophane from indole aldehyde through the intermediate indole pyruvic acid oxime was reported in 1934 by Bauguess and Berg (395). However, neither the quantities nor the percentage yields of intermediate or final products were given. The reactions employed by these investigators are indicated below:

$$\begin{array}{c|c} C-CHO \\ CH \\ CH \\ \hline \\ NH \\ (\beta\text{-3-Indole} \\ \text{aldehyde}) \end{array} \xrightarrow{C_6H_5\cdot CO\cdot NH\cdot CH_2\cdot COOH} \xrightarrow{(CH_3CO)_2O}$$

$$\begin{array}{c|c} C-CH:C-CO & C-CH:C(OH)\cdot COONa \\ \hline CH & O & CH \\ \hline N & N=C-C_6H_5 & NH \\ \hline \\ CO\cdot CH_3 & \end{array}$$

 α -Benzoylamino- β -(1-ace-tyl-3-indole) acrylic acid azlactone

Sodium salt of-α-hydroxy-β-3-indoleacrylic acid

$$\begin{array}{c|c} & C-CH_2 \cdot CO \cdot COOH \\ & HONH_2 \cdot HCl \\ \hline & NH \\ & (\beta\text{-3-Indolepyruvic acid}) \\ \hline & C-CH_2 \cdot C(:NOH) \cdot COOH \\ \hline & H_2 \\ \hline & CH \\ \hline & NH \\ (\alpha\text{-Oximino-}\beta\text{-3-indole propionic acid}) \\ \hline & C-CH_2 \cdot CH(NH_2) \cdot COOH \\ \hline & CH \\ \hline & NH \\ & (Tryptophane) \\ \end{array}$$

Majima's (208) synthesis from indole aldehyde and hydantoin was described in 1922. A 2.8 gm. lot of tryptophane was prepared in an approximately 17 per cent over-all yield. This synthesis has been thoroughly re-investigated by Boyd and Robson (396) in 1935. Because of the poor yields of indole aldehyde obtained by Ellinger and Flamand from indole and chloroform by the Reimer-Tiemann reaction, the preparation of this substance was first investigated. By increasing the quantity of potassium hydroxide reagent the yield of this aldehyde was increased from 8 to 31 per cent of the theoretical amount. Indole-3-aldehyde was also prepared satisfactorily by the following reactions:

$$\begin{array}{c|c} CH: NH \cdot HCl \\ \hline NH \\ \hline \\ CO(OC_2H_5) & \hline \\ CO(OC_2H_5) & \hline \\ CH: NC_6H_5 \\ \hline \\ NH \\ \hline \\ CH: NC_6H_5 \\ \hline \\ CH:$$

As a result of their investigation on the condensation of aromatic aldehydes with hydantoins, these authors found that the yields of hydantoin derivatives could be markedly increased by the use of pyridine as a solvent and diethylamine or piperidine as condensing agent. The yields of products from benzaldehyde, p-methoxybenzaldehyde, and p-hydroxybenzaldehyde were 76, 94, and 65 per cent of the theoretical, respectively. Also, it was discovered that selective reduction of the R-alhydantoins is effected by long continued treatment with ammonium sulfide in ammonium hydroxide at high temperatures. By refluxing indole-3-aldehyde in pyridine with piperidine after 30 minutes the yield of indolalhydantoin was 65 per cent of the theoretical. On reduction of the latter in a closed tube at 100 to 103° with 16 per cent ammonium sulfide and 3 per cent ammonium hydroxide for 500 hours 70 per cent of the theoretical yield of tryptophane was obtained.

Because of the high cost of commercial indole, investigations on the synthesis of this compound have been carried out by M. Friedman and C. E. Redemann in the author's laboratory. Of the methods cited by Hollins (397), Redgrove (398) and others, that described by Majima and co-workers (399) was found to be most satisfactory. Aniline and acetylene were allowed to react at high temperatures with the production of about 18 per cent yield of indole calculated from the aniline decomposed. In small runs, about 20 per cent of the theoretical yields of indole were obtained by the reduction of phenylglycine with sodium-amalgam according to the procedure of Vorländer and Apelt (400).

(21) **Tyrosine.** In 1872, Beilstein and Kuhlberg (401) proposed a synthesis of tyrosine from p-nitro- or p-hydroxycinnamic acid through the intermediate substances α -hydroxy- β -chloro-p-hydroxycinnamic acid, α -hydroxy-p-hydroxycinnamic acid, and α -chloro-p-hydroxycinnamic acid, but only a few preliminary experiments were performed. In the same year, Barth (81) prepared some impure crystals by this method which gave a positive Piria's test for tyrosine. However, difficulties were encountered in carrying out the indicated reactions.

The synthesis of tyrosine from phenylalanine was described by Erlenmeyer and Lipp (79, 110) in 1882. A 2.4 gm. lot of tyrosine was prepared by the reactions which are shown below:

$$\begin{array}{c} \text{C}_{6}\text{H}_{5} \cdot \text{CH}_{2} \cdot \text{CH}(\text{NH}_{2}) \cdot \text{COOH} \xrightarrow{\text{H}_{2}\text{SO}_{4}} \\ \text{(Phenylalanine)} \end{array}$$

$${\rm NO_2C_6H_4\cdot CH_2\cdot CH(NH_2)\cdot COOH} \xrightarrow{\rm Sn+HCl}$$

(p-Nitrophenylalanine)

$$\begin{array}{c} HCl \cdot NH_2 \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2 \cdot HCl) \cdot COOH \\ \hline to \ one \ \ NH_2 \end{array}$$

(p-Aminophenylalanine dihydrochloride)

 $HOC_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

(Tyrosine)

This method is considered to be impracticable because of the simultaneous formation of α -hydroxy-p-hydroxyphenylpropionic acid in the deamination step. The two intermediate compounds were prepared in 98 and 96 per cent of the theoretical yields. A few crystals of a substance which responded to the Hoffman and Piria tests were synthesized by this method by Schulze and Nägeli (402) from phenylalanine. p-Aminophenylalanine dihydrochloride was prepared by Erlenmeyer and Lipp from p-nitrocinnamic ester through the α -nitro-p-cinnamic ester in approximately 20 per cent of the theoretical yield. The attempts of these authors to prepare tyrosine by the reaction of potassium hydroxide on p-sulfonyl-phenylalanine failed.

Tyrosine was synthesized by Erlenmeyer and Halsey (233) from p-hydroxybenzaldehyde and hippuric acid in about 3 per cent of the theoretical yield. Fischer (234) later applied these reactions to the synthesis of 3 gm. of tyrosine in approximately 47 per cent yield calculated from the intermediate α -benzoylamino-p-hydroxycinnamic acid. Because he was not able to obtain a good yield of tyrosine by Erlenmeyer's method, Latham (403) endeavored to improve the synthesis by reducing the intermediate cinnamic acid derivative with a mixture of potassium cyanide, water, and barium hydroxide. A greatly improved synthesis was reported by Harington and McCartney (229) in 1927. By substituting p-methoxybenzaldehyde (anisaldehyde) for p-hydroxybenzaldehyde and, by simultaneously hydrolyzing and reducing the intermediate azlactone with hydriodic acid, red phosphorous, and acetic anhydride, the yield of tyrosine, calculated from ethyl-α-benzoylaminop-methoxycinnamate, was increased to 60 per cent of the theoretical amount. In 1931, Lamb and Robson (230) synthesized tyrosine from anisaldehyde in about 25 per cent of the theoretical yield by the use of glacial acetic acid as the solvent for hydriodic acid and red phosphorous.

In 1911, Wheeler and Hoffman (209) prepared 3.7 gm. of tyrosine from anisaldehyde and hydantoin in approximately 66 per cent of the theoretical yield. This is one of the most satisfactory methods for the synthesis of tyrosine. Procedures for the reduction of anisal- to anisylhydantoin were described by Johnson and coworkers (211, 212), while improvements in the condensation of aromatic aldehydes with hydantoin were made by Boyd and Robson (396).

The synthesis of tyrosine from potassium phthalimidomalonic ester and anisyl bromide was reported in 1914 by Stephen and Weizmann (181), although neither the quantities nor yields of intermediate or final products were stated. However, these reactions have been used satisfactorily for the synthesis of 37 gm. of tyrosine in 34.9 per cent over-all yield in the author's laboratory.

Sasaki (216) synthesized tyrosine in 1921 by the simultaneous reduction and hydrolysis of the condensation product from glycine anhydride and anisaldehyde with hydriodic acid and red phosphorous. A 4.5 gm. lot of tyrosine was prepared in 48 per cent overall yield. This procedure is used routinely in the author's laboratory for the large scale preparation of dl-tyrosine.

(22) Valine. The synthesis of valine from isobutyraldehyde by the Strecker method was reported in 1880 by Lipp (111). Although the yield of amino acid was not given, it seems evident that valine can be synthesized satisfactorily by this method. A synthesis from α -bromo-isovaleric acid and ammonia, first described by Clark and Fittig (133), was investigated by other workers (134–136) and employed by Slimmer (354) for the preparation of valine in 70 per cent of the theoretical yield. This method is used routinely in the author's laboratory for the synthesis of valine from commercial isovaleric acid. The intermediate α -bromo-iso-valeric acid may be prepared from isopropylmalonic ester in 70 to 75 per cent of the theoretical yield by the method of Marvel and du Vigneaud (175). Isopropylmalonic ester has been prepared in 77 to 85 per cent yields (164, 175).

6. ORIGIN OF AMINO ACIDS IN PLANTS AND LOWER ORGANISMS

Yeasts and bacteria possess the power to synthesize amino acids and proteins from inorganic nitrogen compounds. Since the higher forms of animal life cannot utilize nitrogen in inorganic form, they are dependent upon plants and lower organisms for the organic nitrogen compounds which are required for the synthesis and repair of body tissues. While it is evident that amino acids and proteins are synthesized by photochemical action *in vivo* from carbon dioxide, water, and nitrates or ammonia, the steps in this process are undoubtedly complicated and the mechanism of the reactions is at the present time largely a matter of speculation.

That aliphatic, aromatic, and heterocyclic amino acids may be synthesized in vitro from inorganic nitrogen compounds and α -keto acids or reducing sugars has been demonstrated by the experiments of Abderhalden and Rona (404), Tamura (405), Skinner (406), Fromageot and Desnuelle (407), and other workers. In recent investigations by Dhar and Mukherjee (408), the influence of different catalysts and photosensitizers on the rate of formation of amino acids under dark and light conditions has been studied.

A possible synthesis of histidine was postulated by Knoop and Windaus (409) based on experiments by which it was found that methylimidazole was formed from glucose and ammonia by the action of zinc hydroxide and sunlight. According to these authors, the synthesis of histidine *in vivo* might take place by means of the following reaction:

$$\begin{array}{c|cccc} CH-NH & CH=C\cdot CH_2\cdot CH(NH_2)\cdot COOH \\ & CH+NH_2CH_2\cdot COOH \rightarrow NH & N \\ & CH-N & CH \\ (Methylimid- (Glycine) & (Histidine) & H_2O \\ azole) & \end{array}$$

A more general method for the biological synthesis of amino acids from α -keto acids was proposed by Knoop and Oesterlin (238) in 1925. These reactions (shown below) were suggested by the syntheses of amino-n-butyric acid, aspartic acid, glutamic acid, and phenylalanine by the simultaneous hydrogenation and amination of the appropriate α -keto acids in the presence of the catalyst, palladium black. The imino acids, proposed intermediates in these reactions, probably cannot be isolated owing to their instability.

$$\begin{array}{c} \text{R} \cdot \text{CO} \cdot \text{COOH} + \text{NH}_3 + \text{H}_2 \rightarrow [\text{R} \cdot \text{C}(:\text{NH}) \cdot \text{COOH}] \xrightarrow{\text{R} \cdot \text{CO} \cdot \text{COOH}} \\ (\alpha\text{-Keto acid}) & (\text{Imino acid}) \end{array}$$

$$\begin{bmatrix} R & R \\ | & | \\ C = N - C(OH) \\ | & | \\ COOH & COOH \end{bmatrix} \rightarrow$$
(Imino acid)

$$\begin{array}{ccc} & & & & & & \\ R \cdot CH(NH \cdot CO \cdot R) \cdot COOH & & & & \\ & & & & & \\ & & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & & \\ & & \\ & & & \\$$

According to Robinson's (410) theory, cyclic amino acids may be formed by aldol condensations of ammonia, glyoxylic acid, and ketone, according to the following reactions:

$$R \cdot CO \cdot CH_3 + CHO \cdot COOH + NH_3 \rightarrow$$

$$R \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow cyclic structures$$

According to this hypothesis, histidine could be formed according to the following reactions:

2CHO·COOH+CHO·CO·CH₃+3NH₃
$$\rightarrow$$
N—CH
$$CH$$

$$+4H2O+CO2$$
NH—C—CH₂·CH(NH₂)·COOH

Mechanisms for the synthesis of amino acids, alkaloids, purines, and pyrimidines from nitrites, carbon dioxide, and water, through the intermediate compounds, hydroxamic acid (HON:CHOH) and active formaldehyde (H-C-OH), have been proposed by Baly and co-workers (411, 412).

7. SYNTHESIS OF AMINO ACIDS IN THE ANIMAL BODY*

Certain amino acids, e.g., cystine, histidine, lysine, and tryptophane, cannot be synthesized in the animal body according to experiments which have been performed with normal and amino acid-deficient protein diets (413—416) (See Chapter XIX). On the other hand, there is considerable evidence that glycine, alanine,

^{*} According to the most recent investigations of Rose and co-workers [Physiol. Rev., 18, 109 (1938)], only the following amino acids are necessary for the normal growth of the rat: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane and valine.

and several other amino acids may be synthesized by the animal. The dispensability of a given amino acid in the diet of an animal may be determined by the following methods: (a) observations on the growth of animals on normal and amino acid-deficient protein diets; (b) perfusion experiments with surviving animal organs; and (c) the isolation of amino acid derivatives after feeding aromatic compounds incapable of complete oxidation by the body.

It has been shown that glycine may be synthesized in the body by feeding proteins lacking this amino acid and noting that normal growth is maintained. Furthermore, when benzoic acid is fed to a mammal, it is detoxicated by conjugation with glycine to form hippuric acid. From feeding experiments with benzoic acid, it has been found that the glycine which is excreted as hippuric acid is much in excess of that which is present in the dietary protein. Under these conditions, it appears that the extra glycine which is utilized as a conjugating agent has not been formed by the degradation of tissue protein since the total urinary nitrogen is maintained at the normal level (417). However, the marked decrease in urea output indicates that the elements normally used in the formation of this substance were utilized for the synthesis of glycine (418, 419).

The synthesis of glutamic acid has been demonstrated by Sherwin and co-workers (420–422) who found that phenylacetic acid is detoxicated by conjugation with glutamine (monoamide of glutamic acid) to form phenylacetylglutamine (See Chapter V). The synthesis of thyroxine and iodogorgoic acid probably also occurs in the body since these amino acids are not normally contained in the diet.

The synthesis of certain amino acids from keto and hydroxy acids has been reported. Embden and Schmitz (423) showed that tyrosine, phenylalanine, and alanine are formed when the surviving liver of a dog is perfused with solutions containing the α -ketonic acid precursors of these amino acids. A further illustration of this type of synthesis was obtained by Knoop and Kertess (424) who injected the sodium salt of β -phenyl- α -hydroxybutyric acid under the skin of a dog and isolated β -phenyl- α -aminobutyric acid from the urine. Cox and Rose (425) and Harrow and Sherwin (426) successfully substituted dl- β -4-imidazole lactic acid for histidine in the diet of rats. However, not all experiments of this type have been successful. McGinty, Lewis, and Marvel (427) obtained negative results on attempting to substitute α -hydroxy, ϵ -hydroxy, and

 ϵ -amino-n-caproic acids for lysine, while Cox and Rose (425) failed in their efforts to replace histidine with 4-imidazole propionic acid. Also, attempts to demonstrate cystine synthesis (428, 429) in the body by feeding α -dihydroxy- β -dithiodipropionic acid and other sulfur containing compounds gave negative results.

Embden's perfusion experiments on the synthesis of alanine from ammonium pyruvate seem to have been confirmed by the recent investigations of Neber (430) and Krebs (431). The former worker found that large amounts of amino nitrogen are formed when ammonium pyruvate is added to surviving slices of rat liver. Although these results were corroborated by Krebs with rat and bird livers, amino nitrogen formation was not observed when pyruvic acid was replaced by α -ketoglutaric and other α -keto acids. This result supports Knoop's (424, 432) hypothesis that pyruvic acid plays a special rôle in the synthesis of amino acids, as shown by the following equations:

 $R \cdot CO \cdot COOH + NH_3 + CH_3 \cdot CO \cdot COOH \rightarrow R \cdot CH(NH \cdot CO \cdot CH_3) \cdot COOH \rightarrow$

 $R \cdot CH(NH_2) \cdot COOH + CH_3 \cdot COOH$

As stated by Krebs (431), this theory was based on the isolation of acetylamino acids from urine and tissues, the utilization of acetylamino acids in the organism, and on analogous reactions in vitro. According to du Vigneaud and Irish (433), Knoop and Blanco's (434) abandonment of this theory was based on a misconception with regard to the configuration of the acetylamino derivative.

Convincing evidence has been presented recently by du Vigneaud and Irish (433) in favor of Knoop's early acetyl theory for the *in vivo* synthesis of an amino acid from the corresponding keto acid.

Dakin and Dudley (435) have stressed the possible importance of the glyoxals in the intermediary metabolism of the amino acids. Although *l*-leucine was obtained when a liver was perfused with isobutyl glyoxal, alanine could not be isolated from the perfusion fluid to which methyl glyoxal had been added. Glycollic acid was found when glyoxal was perfused through a liver, but the presence of glycine could not be conclusively demonstrated. The probable importance of methyl glyoxal in the animal body is indicated by the following equilibria which are thought to be established:

$$C_6H_{12}O_6 \text{ (Glucose)}$$

$$CH_3 \cdot CH(OH) \cdot COOH \rightleftharpoons CH_3 \cdot CO \cdot CHO$$

$$CH_3 \cdot CH(NH_2) \cdot COOH \text{ (Alanine)}$$
 (Lactic acid) (Methyl glyoxal)

Glyoxals may be intermediates in the conversion of amino acids into alcohols by the action of yeast, as indicated by the following illustration:

$$\begin{aligned} \mathrm{NH_2 \cdot CH_2 \cdot COOH} &\rightleftharpoons \mathrm{NH_3 + CHO \cdot CHO} &\rightleftharpoons \mathrm{CH \, (OH) : CO} & \stackrel{H_2\mathrm{O}}{\Longleftrightarrow} \mathrm{CO_2 + \, CH_3OH} \\ & \text{(Glycine)} \end{aligned} \end{aligned}$$

8. AMINO ACIDS REPORTED BUT NOT VERIFIED

Amino acids which have been reported to be constituents of proteins, but not verified in accordance with the criteria discussed on pages 21 and 22, are presented in this section. It appears altogether probable that a number of these amino acids may be added to the list of accepted amino acids. While the fact that not all of the nitrogen of proteins is accounted for by the amino acids which have been isolated may be due to inaccuracies in the analyses, it seems probable that this condition may be explained by the existence of unidentified amino acids. As the technique for the isolation and identification of amino acids becomes more refined, it may be expected that additional forms may be brought to light.

(1) Aminobutyric acid or α -amino-n-butyric acid.

$$\mathrm{CH_3}\cdot\mathrm{CH_2}\cdot\mathrm{CH(NH_2)}\cdot\mathrm{COOH}$$

The isolation of this amino acid from the alkaline hydrolysate of silk fibroin was announced by Schützenberger and Bourgeois (436) in 1875. However, a reinvestigation by Fischer and Skita (437) in 1901 showed that this conclusion was incorrect. A product thought to be aminobutyric acid was isolated by Foreman (438) from casein, but the melting point of this substance was lower than the value reported by Fischer and Mouneyrat (439) for synthetic aminobutyric acid. The isolation of this amino acid from proteins was also reported by Abderhalden and Weil (61) in 1912, but no experimental proof was given.

(2) Canavanine or α -amino- γ -guanidinoxy-n-butyric acid

 $NH_2 \cdot C(:NH) \cdot NH \cdot O \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

The isolation of canavanine from soy bean meal was reported by Kitagawa and Monobe (440) in 1933. The constitution proposed by Kitagawa and co-workers (441) was confirmed by Gulland and Morris (442) who obtained glyoxal and tartronic semialdehyde by oxidation with Chloramine T and ammonia, guanidine, and α -amino- γ -butyrolactone by treatment with hot hydrobromic acid. The latter authors described an improved method for the isolation of canavanine. The melting point was shown to be 184° and $[\alpha]_{n}^{20}$, +7.90. Tomiyama (443) isolated canavanine from Jack bean meal and converted it to urea and canaline [NH₂·O·CH₂-CH₂·CH(NH₂)·COOH by the action of liver ferment. The dissociation constants of canaline and canavanine were determined and it was shown that the guanidine group of the latter compound is weaker than that of arginine, but stronger than that of the imidazole group. The synthesis of canaline and canavanine has been described recently by Kitagawa and Takani (444).

(3) Citrulline or δ -carbaminoornithine.

 $NH_2 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$

This amino acid was isolated by Wada from watermelon juice (445) and casein (446). Ackerman (447) obtained citrulline by the action of bacteria on arginine, while Dirr and Späth (496) converted arginine into citrulline by amidation, benzoylation, and hydrolysis. From Wada's investigations it was shown that proline is obtained from citrulline by the action of acids, and ornithine is obtained, when it is treated with bases. The synthetic product, prepared by the following reactions, was shown to resemble the inactivated natural substance:

 $NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow$ (Ornithine)

 $\begin{array}{c} \mathrm{C_6H_5 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5) \cdot COOH} \\ \\ \mathrm{(Dibenzoylornithine)} \end{array}$

 $\begin{array}{c} NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5) \cdot COOH \rightarrow \\ (\alpha\text{-Monobenzoylornithine}) \end{array}$

 $NH_2 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH \cdot CO \cdot C_6H_5) \cdot COOH \rightarrow$ $(\alpha-Monobenzoyl-\delta-carbaminoornithine)$

$$NH_2 \cdot CO \cdot NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$$
 (Citrulline)

A simple synthesis of dl-citrulline from dl-ornithine that utilizes the capacity of copper to form complexes with the two substances has been described recently by Kurtz, A. C., [J. Biol. Chem., 122, 477 (1938)].

(4) Djenkolic acid

$$CH_2[S \cdot CH_2 \cdot CH(NH_2) \cdot COOH]_2$$

An amino acid of the empirical formula, $C_7H_{14}N_2S_2O_4$, and of the foregoing proposed structure, was isolated from djenkol nuts and from urine by Van Veen and Hyman (448) in 1935. Cystine and formaldehyde were obtained by sulfuric acid hydrolysis and the following properties were determined: melting point, about 250°; $[\alpha]_D^{20}$, -25° for 2 per cent solution in 1 per cent hydrochloric acid; and melting point of dibenzoylderivative, 85°. Djenkolic acid has been synthesized by du Vigneaud and Patterson (499) by permitting cysteine to react with methylene dichloride in liquid ammonia.

(5) 3:5-Dibromotyrosine.

$$\begin{array}{c} \text{Br} \\ \text{HO} \\ \hline \\ \text{Br} \end{array} \\ \text{CH}_2 \cdot \text{CH(NH}_2) \cdot \text{COOH} \\ \end{array}$$

This amino acid was isolated from the horny skeleton of the coral, *Primnoa lepadifera*, by Mörner (449). The synthesis from tyrosine and bromine was described by Zeynek (450) in 1921. The identification of this substance, by means of the properties listed by Gorup-Besanez (451) and the foregoing authors, is so complete that there is little doubt concerning its existence in certain special proteins.

(6) Dihydroxyphenylalanine or α -amino- β -3:4-dihydroxyphenylpropionic acid.

$$H$$
 O
 $CH_2 \cdot CH(NH_2) \cdot COOH$

This amino acid, first found by Torquati (452) in aqueous extracts of the pods and sprouts of *Vicia faba*, also occurs in the velvet bean as shown by Miller (453). Dihydroxyphenylalanine has not been isolated from protein hydrolysates probably because of

its ease of oxidation. It is best synthesized by condensing vanillin with hippuric acid (229), resorcinol aldehyde with glycine anhydride (454), or piperonyl bromide with ethyl phthalimidomalonate (181). Properties of the synthetic substance have been determined by Waser and Lewandowski (455) and Sugii (456). The identity of the natural with the synthetic amino acid, first prepared by Funk (353), has been demonstrated by Guggenheim (457) and Harington and Randall (459). According to Raper (458), dihydroxyphenylalanine is the first stage in the formation of melanin from tyrosine.

(7) Threonine or l- α -amino- β -hydroxy-n-butyric acid (504).

$$\mathrm{CH_3}\!\cdot\!\mathrm{CH}(\mathrm{OH})\!\cdot\!\mathrm{CH}(\mathrm{NH_2})\!\cdot\!\mathrm{COOH}$$

This amino acid was first isolated from oat protein by Schryver and Buston (460), from teozin by Gortner and Hoffman (461), and from casein by Rimington (462) and Czarnetzky and Schmidt (463). However, none of these workers determined the constitution of this hydroxyaminobutyric acid.

In a series of outstanding investigations, Rose*and his co-workers (465-468) have isolated l- α -amino- β -hydroxy-n-butyric acid in considerable quantity from proteins, determined its physical and chemical properties, shown that it is an indispensable dietary constituent, and established its identity with the synthetic material. The isolation of a 4.5 gm. lot of this amino acid was accomplished by hydrolyzing 12 kg. of commercial fibrin with sulfuric acid, removing the less soluble amino acids, fractionally crystallizing the more soluble amino acids as their copper salts, removing amino acids with no growth-promoting activity by extraction with n-butyl alcohol, and precipitating the desired product with phosphotungstic acid and ethyl alcohol. It was deduced from the chemical and physical tests on the purified material that the unknown amino acid was one of the possible isomeric hydroxyaminobutyric acids. The final conclusion that the new substance is one of the four optically active α -amino- β -hydroxy-n-butyric acids was confirmed by experiments on the synthetic product. The following reactions were employed by Carter and co-workers (464) in their synthesis of this amino acid:

$$\begin{array}{c} \mathrm{CH_3 \cdot CH : CH \cdot CO(OC_2H_5)} \xrightarrow{\mathrm{Hg(O \cdot OC \cdot CH_3)_2}} \\ \mathrm{(Ethyl\ crotonate)} \end{array}$$

^{*} See Rose, W. C., Physiol. Rev., 18, 109 (1938), for a review of recent work on the nutritive significance of the amino acids.

$$\begin{array}{c} \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OCH}_3)\cdot\operatorname{CH}(\operatorname{HgO}\cdot\operatorname{OC}\cdot\operatorname{CH}_3)\cdot\operatorname{CO}(\operatorname{OC}_2\operatorname{H}_5) \xrightarrow{\operatorname{KBr}} \\ & (\operatorname{Mercuric acetate-ethyl crotonate complex}) \\ \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OCH}_3)\cdot\operatorname{CH}(\operatorname{HgBr})\cdot\operatorname{CO}(\operatorname{OC}_2\operatorname{H}_5) \xrightarrow{\operatorname{Br}_2} \\ & (\operatorname{Intermediate}) \\ \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OCH}_3)\cdot\operatorname{CHBr}\cdot\operatorname{CO}(\operatorname{OC}_2\operatorname{H}_5) \xrightarrow{\operatorname{NaOH}} \\ & (\operatorname{Ethyl-}\alpha\text{-bromo-}\beta\text{-methoxybutyrate}) \\ \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OCH}_3)\cdot\operatorname{CHBr}\cdot\operatorname{COONa} \xrightarrow{\operatorname{H}_2\operatorname{SO}_4} \\ & (\operatorname{Sodium\ salt\ of\ }\alpha\text{-bromo-}\beta\text{-methoxybutyric\ acid}) \\ \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OCH}_3)\cdot\operatorname{CHBr}\cdot\operatorname{COOH} \xrightarrow{\operatorname{NH}_3} \\ & (\alpha\text{-Bromo-}\beta\text{-methoxybutyric\ acid}) \\ \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OCH}_3)\cdot\operatorname{CH}(\operatorname{NH}_2)\cdot\operatorname{COOH} \xrightarrow{\operatorname{HBr}} \\ & (\alpha\text{-Amino-}\beta\text{-methoxy-}n\text{-butyric\ acid}) \\ \operatorname{CH}_3\cdot\operatorname{CH}(\operatorname{OH})\cdot\operatorname{CH}(\operatorname{NH}_2)\cdot\operatorname{COOH} \\ & (\alpha\text{-Amino-}\beta\text{-hydroxy-}n\text{-butyric\ acid}) \\ \end{array}$$

These reactions, first reported by Abderhalden and Heyns (469), were found by B. Rosenberg in the author's laboratory to give good yields in all but the final step.

Since this synthetic mixture showed little, if any, growth stimulation, the pair of mirror image isomers obtained by the synthesis was racemized to a mixture of the four isomers. The latter showed about one-fourth to one-sixth of the activity of the natural amino acid. Epimerization was accomplished by heating the formyl derivative of α -amino- β -methoxy-n-butyric acid with sodium hydroxide, water, and acetic anhydride, according to the method of du Vigneaud and co-workers (281, 282).

As the result of recent investigations by Meyer and Rose (470), it has been determined that the naturally occurring hydroxyamino-butyric acid is an L-D type corresponding in spatial structure to d(-)-threose. Hence, it has been proposed that henceforth natural α -amino- β -hydroxy-n-butyric acid be known as d(-)-threonine. The configuration of these molecules is shown below:

The experiments of Rose and co-workers represent the first successful attempt to induce growth in animals upon diets with synthetic mixtures of highly purified amino acids in place of proteins. Also, it is of interest that β -hydroxyglutamic acid and citrulline were found to be dispensable components of the diet.

(8) Hydroxylysine or $\alpha - \epsilon$ -diamino- β -hydroxy-n-caproic acid.

$$NH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(OH) \cdot CH(NH_2) \cdot COOH$$

A base which could be precipitated as its phosphotungstate and mercury salt from gelatin hydrolysate, was reported by Schryver and co-workers (471) in 1925. Two years later, Schryver and Buston (472) isolated this substance from a number of proteins. This base was not precipitated by silver salts from solutions made alkaline with barium hydroxide. Hence, it could be separated from arginine and histidine. The free base was hygroscopic and absorbed carbon dioxide from the air. Both the copper salt and the nitrate were quite deliquescent. Since this amino acid has not been synthesized, its constitution has not been established. See (503).

(9) Hydroxyvaline or α -amino- β -hydroxy-isovaleric acid.

$$(CH_3)_2 \cdot C(OH) \cdot CH(NH_2) \cdot COOH$$

Hydroxyvaline was isolated from oat protein by Schryver and Buston (460) and later by Czarnetzky and Schmidt (473). Brazier (474) reported its presence in zein. The structure of this amino acid has not been determined by synthesis. Abderhalden and Heyns (502) have not been able to confirm the presence of this amino acid in proteins.

(10) Norvaline or α -amino-n-valeric acid.

$$CH_3 \cdot CH \cdot CH_2 \cdot CH(NH_2) \cdot COOH$$

Evidence that norvaline is a constituent of globin, casein, and steer-horn has been presented by Abderhalden and co-workers (64, 475, 476). It was shown that natural norvaline and synthetic valine formed hydantoins of different melting points. However, the identity of this amino acid was more definitely established by a method which differs in principle from others previously used. The valine-leucine fraction, obtained by the ester distillation method, was fractionally crystallized and a valine fraction isolated. The latter was converted to α -bromo-valeric acid by the action of nitrosyl bromide. This product was treated with ammonia or trimethylamine (477) and the rate at which bromide ion was formed was determined. This ratio was compared with the rates at which bromine was removed from synthetic α -bromo-n-valeric acid and α -bromo-isovaleric acid from natural valine. The curve for the amino acid isolated from proteins resembled that from α -bromo-n-valeric acid.

(11) Thiolhistidine or 2-thiol- β -imidazole- α -aminopropionic acid.

$$\begin{array}{c} \text{NH--C--CH}_2\text{-}\text{CH}(\text{NH}_2)\text{-}\text{COOH} \\ \\ \text{HS-C} \\ \\ \text{N---CH} \end{array}$$

The evidence for the occurrence of this amino acid in proteins is indirect. Ergothionine, the betaine of thiolhistidine, has been shown to occur in animal blood by Eagles and Johnson (478), Newton and co-workers (479), and Hunter (480). Eagles and Vars (481) found that ergothionine is absent from pig's blood when casein is the sole source of protein, while it re-appeared when the animals were fed corn. Tanret found this substance in ergot. It was synthesized by Barger and Ewins (482). Syntheses of thiolhistidine from histidine and from aspartic acid have been reported by Harington and co-workers (483, 484).

(12) Protoctine.

A product of the composition C₈H₁₅O₃N₃ was isolated from oat and castor bean proteins by Schryver and Buston (485). While the constitution of this substance was not definitely determined, the amino acid was found to contain one amino, one carboxyl, and one hydroxyl group.

(13) Tribasic amino acid from liver.

$$\begin{array}{c|c} CH_2-CH-CH_2\cdot CH_2\cdot CH(COOH)_2\\ \\ HOOC\cdot CH_2\cdot CH & CO\\ \\ NH \end{array}$$

The isolation of this amino acid from liver extract was reported by Dakin and West (486) in 1931. While the constitution of this substance was not definitely established, it was shown to contain three carboxyl groups but no amino nitrogen. This amino acid appears to be closely related to pentane- β - λ - ϵ -tricarboxylic acid [CH₃·CH(COOH)·CH(COOH)·CH₂·CH₂·COOH], a substance which has been obtained by the reduction of tribasic hematinic acid derived from hematin.

- (14) Miscellaneous amino acids. Additional amino acids which have been reported by various authors are listed under this heading. On account of the lack of convincing evidence that these compounds are constituents of proteins, they are discussed only briefly.
- (a) Tetratrisäure. The isolation from hemoglobin digest of a compound containing four amino and three carboxyl groups was reported by Fränkel and Monasterio (487) in 1929. The composition of this substance was found to be $C_{22}H_{46}N_4O_7$.
- (b) Dihydroxypyrrole-alanine. After the removal of the basic amino acids from the phosphotungstate precipitate obtained with casein and other protein hydrolysates, Van Slyke and co-workers (488, 489) isolated a substance whose ratio of total to amino nitrogen was 2:1. The composition of the copper salt was found to be $(C_7H_9O_4N_2)Cu$ and the product gave a test for the pyrrole group. It was concluded from these data that the substance was dihydroxypyrrole-alanine. However, Emerson and Schmidt (490) could not confirm this evidence.
- (c) Basic amino acid from Jack bean. Kitagawa and co-workers (491, 492) isolated a product of the composition $C_5H_{12}N_4O_3$ from the non-protein fraction of Jack bean extract. The substance is basic, is precipitated by phosphotungstic acid, and reacts with a liver enzyme to liberate one-half of its total nitrogen as urea.
- (d) Diaminotrioxydodecanic acid. Fischer and Abderhalden (493) described the isolation of a substance which had the composition C₁₂H₂₆N₂O₅. However, in 1917, Fischer (494) stated that the individuality of the product was doubtful.
- (e) In 1904, Skraup (495) reported the isolation from casein of the following products: diaminoglutaric acid, C₅H₁₂O₄N₂; diaminoadipic acid, C₆H₁₄O₄N₂; hydroxyaspartic acid, C₄H₇O₅N; dioxydiaminosuberic acid, C₈H₁₆N₂O₆; a tribasic acid, caseansäure, C₉H₁₆N₂O₆; and a dibasic acid, caseinic acid, C₁₂H₁₆N₂O₅. At the present time, the evidence for the existence of these substances seems inacceptable.

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CHAPTER III

SECTION I. THE ISOLATION OF THE AMINO ACIDS FROM PROTEINS

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1. INTRODUCTION

The classification of the proteins occurring in both plants and animals according to their origin, solubility, coagulability, and other physical properties, has been of inestimable value for certain types of investigations but furnishes very little information concerning their chemical constitution. The same can be said of the results of elementary analyses. That the composition of complex compounds cannot be thus ascertained was first recognized by Liebig. He stated that this can be done only by analysis of the decomposition products. Elementary analysis aids only in distinguishing proteins which contain some special element such as the iron of hemoglobin, the phosphorus of casein or the copper of hemocyanin. Precipitation reactions are too general to be of value. Color reactions may distinguish certain groups but most of these are characteristic constituents of proteins in general, and further, when applied directly, are not quantitative.

Investigations of the composition of proteins have been carried on for well over a century, but it is only within the last two or three decades, due to the work of a large number of investigators, among them Hofmeister, Kühne, Kossel, Fischer, Sörensen, Svedberg, Van Slyke and others, that any clear concept has really been obtained of the structure of proteins. The result of these studies has led to the concept that the amino acids are linked through the amino group of one to the carboxyl group of the other with the loss of water, thus forming a substituted amide; further, that secondary valences which are present at the various points cause the molecule to assume a definite pattern which is a characteristic of each protein (See Chapter VII).

2. HYDROLYSIS

The method of hydrolysis is the one which has contributed most to our present knowledge regarding the structure and chemical composition of the protein molecule. Hydrolysis has been effected primarily by three methods, (1) boiling with acids, (2) boiling with alkalies, and (3) the action of the proteolytic enzymes. Under the conditions of the experiments it is doubtful if any one of these methods ever produces complete hydrolysis, although a point may be reached where the number of amino and carboxyl groups is at a maximum.

Of the three methods, boiling the protein with strong acids is the one which is most used to effect hydrolysis. When this is done, proteins yield a mixture of α -amino acids, as well as ammonia and such non-protein prosthetic groups as may be present in the particular protein under investigation. The technical difficulties involved in a quantitative separation of the split products are so great that a high summation of amino acids can hardly be expected. In fact, a large number of summations of amino acids of various proteins have yielded results ranging from 65 to 85 per cent. For gelatin 91.3 per cent, and for zein 102 per cent have been found; 110 to 120 per cent would represent complete recovery due to the fact that water molecules are added during the hydrolysis. In spite of the difficulties, the optimism of many workers is represented in a quotation from Vickery (12) "It seems highly improbable that any appreciable amount of a hitherto unknown amino acid can occur in zein, and the day does not seem far distant when we shall know the composition of the whole of the molecule of at least one protein." It may, however, be pointed out that even if it were possible to obtain a complete analysis, our knowledge of the constitution of proteins would still be very incomplete.

(1) Sulfuric Acid. Certainly from an historical standpoint and probably also from a practical standpoint, sulfuric acid holds first place among the acids that have been used to hydrolyze proteins. The first amino acid isolated from a protein hydrolysate was glycine. It was obtained by Braconnot (13) in 1820 from sulfuric acid hydrolysates of gelatin and also of meat. Sulfuric acid has subsequently been used by almost every investigator of protein chemistry. The concentration of sulfuric acid usually used is approximately 35 per cent. The mixture of protein and acid is first heated on a steam bath from 1 to 3 hours until frothing ceases and subsequently refluxed gently on an oil or an air bath (105° to 110°)

until the hydrolysis is complete, as determined by either the Van Slyke or the Sörensen method. The time required is usually 12 to 24 hours. Sometimes a longer period may be necessary or it may be necessary to autoclave the mixture at 135° to 150° for a short time depending upon the protein which is being investigated. The autoclave is seldom used and it is very doubtful if it has any advantage over a longer period of heating at ordinary pressures. The principal advantage in the use of sulfuric acid rather than hydrochloric acid is the ease with which the sulfate ion may be removed by barium hydroxide, or barium hydroxide followed by barium carbonate.

(2) Hydrochloric Acid. A reagent frequently used for the hydrolysis of proteins is hydrochloric acid. It was first introduced in 1868 (14) for the hydrolysis of vegetable proteins. Sulfuric acid is generally preferred if the butyl alcohol procedure is to be followed or the bases are to be isolated. Hydrochloric acid is preferable when the monoamino acids are to be determined. Hydrolysis with hydrochloric acid is usually carried out with 3 to 5 parts of concentrated acid or 10 to 20 parts of 20 per cent acid. Complete hydrolysis may also be obtained with 3 N acid in an autoclave at 150° for 1 to 2 hours. The time required for the usual hydrochloric acid hydrolysis is 6 to 24 hours when the mixture is gently refluxed on an oil bath. Some proteins, however, may require 48 hours. Casein is one of those that is difficult to hydrolyze completely. Hydrochloric acid hydrolyses have, at times, been carried out in the presence of stannous chloride. The solution, which otherwise turns dark brown or black, remains colorless (15). Fischer and others have not considered stannous chloride necessary, especially with certain proteins, even when strong acid is used (16).1

According to Van Slyke (17), the percentage of amino nitrogen reaches a definite maximum when the acid hydrolysis of a protein is complete, and this maximum is the same whether the hydrolysis occurs at 100° or at 150°. If the proteins were boiled with from 10 to 20 parts of 20 per cent hydrochloric acid for 48 hours, constant results were obtained. In no case did the amino nitrogen reach a maximum in 10 hours when the hydrolysis was carried out at 100°. Twenty four hours were adequate in all cases except in the case of gluten and casein which required 48 hours. Further hydrolysis often

¹ According to Sullivan, M. X., and Hess, W. C., J. Biol. Chem., 117, 423 (1937), addition of titanous chloride prevents the formation of humin. The titanium can be removed by neutralization with sodium hydroxide to pH not greater than 6.0.

caused a slow but definite decrease in the value for amino nitrogen, while the amount of ammonia never reached a definite maximum, but slowly increased the longer the hydrolysis was continued. This was attributed to a slow but definite decomposition of amino acids by the boiling strong acid used in the hydrolysis.

Various concentrations of hydrochloric as well as sulfuric acid have been used in hydrolyzing proteins in an autoclave. Alcoholic hydrochloric acid has also been used, but it offers no obvious advantages. Several other acids have been tried, including hydrofluoric acid, hydrobromic acid, hydriodic acid, formic acid, phosphoric acid, and acetic acid, as well as acetic anhydride. No one of them has any definitely established advantage except possibly when it is desired to isolate a particular amino acid.

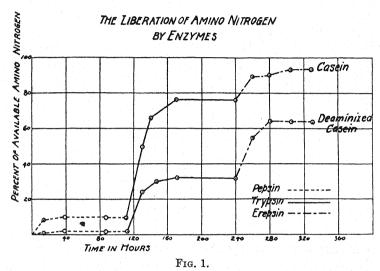
Greenberg and Burk (18) and Nasset and Greenberg (19) have studied the effect of temperature and concentration of acid in hydrolyzing proteins. The catalytic effect of acids on protein hydrolysis was found to be proportional to the thermodynamic activity of the hydrogen ion. The influence of temperature can be expressed by the equation,

 $\log K_a = 0.0287T - 5.30$,

where K_a refers to the velocity constant at unit activity, and T is expressed in degrees Centigrade. The increase in amino nitrogen with time follows the equation for a second-order reaction.

- (3) Hydroxides. The alkalies, sodium hydroxide, potassium hydroxide, and barium hydroxide, produce rapid and complete hydrolysis of proteins, but have the advantage over acids only in that they do not destroy tryptophane. Hence they are often used when this amino acid is to be estimated or isolated. Tyrosine also is not destroyed by alkali and can be determined in the same hydrolysate. Serine is destroyed by prolonged treatment with alkali. Thyroxine is best liberated by mild alkaline hydrolysis of the thyreoglobulin of the thyroid gland. Hydrolysis of proteins with ammonium hydroxide or ammonium carbonate under pressure has also been accomplished but offers no advantages.
- (4) Enzymes. The third group of hydrolytic agents consists of the proteolytic enzymes. Although the writer hopes that the new nomenclature for the tryptic and ereptic systems of enzymes will be adopted by textbooks as rapidly as possible, it is necessary that the old terminology be used here since most of the studies recorded in the literature were carried out with mixtures of enzymes and not with the individual enzymes.

The study of the action of the vegetable enzymes on proteins was initiated and continued by Schulze and his co-workers. Kühne, Chittenden, Kossel, Kutscher, Drechsel, Willstätter, Waldschmidt-Leitz, Northrop, Calvery, and others (20, 21) have studied the action of animal enzymes on many different proteins. The hydrolytic action of the proteolytic enzymes on protein is probably never complete, but in some instances may be as nearly so as is alkaline or acid hydrolysis (21). Early investigators reported the



(Dunn, M. S., and Lewis, H. B., J. Biol. Chem., 49, 345 (1921).)

presence of resistant groups which usually contained all of the phenylalanine and proline of the protein molecule (22). The combined action of pepsin and trypsin or the enzymes of the small intestine accomplishes almost complete hydrolysis if the reaction is allowed to continue for a sufficient length of time. (See Fig. 1 and Table I).

The mild conditions of hydrogen and hydroxyl ion concentrations and of temperature under which enzymic hydrolysis of proteins proceeds is of great advantage in investigations of the content of amino acids in proteins, since the treatment is much less likely to produce destruction or change of these units. This is particularly true when it is desired to determine the optical activities of the resulting amino acids.

Since the various methods mentioned above are at present the only means of attack on the structure of the protein molecule, it is

TABLE I

Hydrolysis by Pepsin, Trypsin, Papain, Protaminase, Carboxypolypeptidase, Aminopolypeptidase, and Erepsin

Sequence is given and the amount calculated as amino nitrogen in per cent of total nitrogen. Calculation is made on the basis of 200 mg. of ash- and moisture-free crystalline egg albumin. Increase in —COOH groups was determined by titration in alcohol and the increase in amino groups by the method of Van Slyke. Both are calculated in cc. of 0.2 N acid. Trypsin = pure pancreatic proteinase.

Experi- ment No.	Sequence of enzymes	Increase (cc. 0		Amino N of total N	Probable peptide linkages hydro-	Probable percent- age of
No.		соон	$\mathrm{NH_2}$	total N	lyzed	splitting
				per cent		
1	Pepsin Trypsin	0 2.70	2.81	25 0	90	24
$\overline{2}$	Pepsin	2.70	2.81	25	90	24
	Papain-HCN		1.86	20	72	20
	Sum		4.67	45	162	44
3	Pepsin	2.70	2.81	25	90	24
	Protaminase	0.88	0.89	7	25	7
	Aminopolypeptidase	2.12	2.00	18	65	17
- 121 - 1	Sum	5.70	5.70	50	180	48
4	Pepsin	2.90	2.84	25	90	24
	Protaminase + carboxy- polypeptidase	2.67	2.70	25	90	24
	Sum	5.57	5.54	50	180	48
5	Pepsin	2.70	2.81	25	90	24
	Aminopolypeptidase	2.73	2.81	25	90	24
	Sum	5.43	5.62	50	180	48
6	Pepsin	2.70	2.81	25	90	24
	Protaminase	0.88	0.89	7	25	7
	Erepsin	4.18	4.13	38	155	41
	Sum	7.76	7.83	70	270	72
7	Pepsin	2.70	2.81	25	90	24
	Erepsin	4.99	5.10	45	180	48
	Sum	7.69	7.91	70	270	72
8	Acid hydrolysis			72.80	270	72

(Calvery, H. O., J. Biol. Chem., 102, 83 (1933).)

essential to point out some of the advantages and the shortcomings of these methods. There is little choice between hydrochloric and sulfuric acid. Hydrochloric acid is usually used when the monoamino monocarboxylic acids and the dicarboxylic acids are to be estimated. Sulfuric acid is used when the butyl alcohol method is employed to separate the monoaminomonocarboxylic acids from the other amino acids. Either acid may be used with success when the basic amino acids are to be determined. Hydrolysis may be carried to the same degree of completeness with either acid. It should be borne in mind, however, that one acid is volatile while the other is not, so that the sulfate ion must be removed by pre-

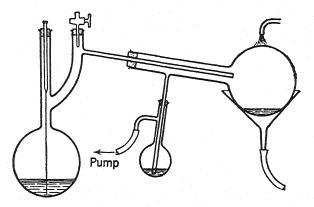


Fig. 2. Arrangement of Claissen flask for concentrating protein hydrolysates in vacuo.

cipitation, while a large percentage of the hydrochloric acid may be removed by vacuum distillation (See Fig. 2), and the remainder by precipitation with silver oxide in the presence of sulfuric acid. Silver sulfate or silver nitrate may also be used for this purpose. When silver nitrate is used, the nitrate ion cannot be subsequently removed. The hydrolysis with hydrochloric or sulfuric acid always involves practically complete destruction of tryptophane, some racemization of other amino acids, and, in the case of most proteins, the formation of considerable quantities of black or brownish-black humin (2). The removal of chloride ions from the hydrolysates with silver salts involves some destruction of cystine (23), while the removal of the sulfate ion involves large losses by adsorption or mechanical incorporation of large amounts of the amino acids in the bulky precipitate of barium sulfate. For example, the writer has noted at times that as much as 15 to 20 per

cent of the total nitrogen may be incorporated in the voluminous precipitate of barium sulfate before it is washed. Subsequent washing by trituration with boiling water will remove most of this and hot dilute acid will remove more, but 100 per cent recovery is seemingly impossible, the principal loss being in the tyrosine and the dicarboxylic amino acid fractions.

The principal advantage accruing from the use of alkalies as hydrolytic agents, namely, the failure of destruction of tryptophane, has already been mentioned. No other distinct advantages can be enumerated, although a small amount of evidence has been presented that alkalies hydrolyze proteins more completely than do acids. The disadvantages in the use of alkali are the complete destruction of cystine, the conversion of arginine into ornithine, the racemization of most of the amino acids, the deamination of some of these, and the loss of ammonia during the hydrolysis.

The advantages from the use of enzymes have already been mentioned. The disadvantages are slowness of hydrolysis, incompleteness of hydrolysis in many cases, and the presence of the enzymes and their products in the resulting hydrolysates. The last factor constitutes the most serious disadvantage in the use of enzymes as hydrolytic agents since it is impossible to separate or distinguish the enzymes and the products which are formed as the result of their hydrolysis from those resulting from the enzymic action on the protein under investigation. The recent advances in the purification of enzymes will undoubtedly lead to their wider use in the study of proteolytic hydrolysis-products. Even now enzymes are often used for the isolation of tryptophane, tyrosine, and, at times, other amino acids.

(5) Humin Formation. The hydrolysis of proteins with strong acids usually produces a black or brownish-black amorphous precipitate which is called melanin or humin. Two proteins which yield very little or no melanin are zein and pepsin. A large number of investigations (2) as to possible precursors of the melanins has led to the theory that they are formed primarily by the condensation of the tryptophane with an aldehyde. The reaction is apparently at the α -hydrogen of the indole nucleus. The nature of the aldehyde group or other groups which react like aldehydes in condensing with the tryptophane to form melanin, is unknown. When zein, a protein which yields only small amounts of melanin, and which contains no tryptophane or carbohydrate, is heated with tryptophane and carbohydrate, nearly all of the tryptophane

nitrogen is obtained in the form of melanin (2). In the case of fibrin, it has been found that a maximum amount of melanin can be formed during the hydrolysis by the addition of a definite amount of formaldehyde. The suggestion has been made that the experiment can be so carried out as to furnish a quantitative measure of the tryptophane content of the protein. The suggestion, however, has not been followed further. Due to the variable amount of tryptophane and of carbohydrate in the proteins, the melanin nitrogen bears little or no quantitative relationship to either one.

Another theory of melanin formation is that this substance is composed of oxidation products of amino acids. There is very little evidence to support this idea. With regard to these substances in general, a quotation from Mitchell and Hamilton (p. 104) seems quite appropriate, "Most of the accumulated data, however, point to the conclusion that the artificial humin formed during the acid hydrolysis of proteins is not a simple condensation product, but is due to a condensation followed by rearrangement or oxidation or both, with the ultimate formation of an extremely resistant molecule or molecules. The nature of the chemical reactions involved and the structural configuration of the humin molecule or molecules formed still require elucidation." "The formation of humin, therefore, interferes to a considerable extent in the analysis of the protein molecule. Of the total nitrogen in the protein, usually from 1 to 2 per cent is found in the insoluble humin. Another loss is due to the soluble brown humin which colors the solution. Tryptophane especially is lost in the insoluble humin fraction while tyrosine, at least, is concerned in the formation of the soluble humin."

(6) Formation of Ammonia. When an acid hydrolysate of most proteins is neutralized, subsequently rendered alkaline with some mild alkali such as magnesium oxide or calcium hydroxide, and then distilled under reduced pressure into standard acid, the ammonia is quantitatively collected and can be determined. The amount produced depends entirely on the protein under investigation. Since the amino acids which result from protein hydrolysis, under the same treatment, do not yield more than traces of ammonia (2), this compound must originate from some form of nitrogen in the protein molecule other than that which is present in free amino acids. It is very probable that the ammonia or amide nitrogen originates from the acid amide, $-\text{CONH}_2$, groups of the dicarboxylic amino acids. A considerable number of the carboxyl groups of the dicarboxylic amino acids, aspartic, glutamic, and β -hydroxy-

glutamic acid, which are not combined in the protein molecule as peptide linkages, are present in proteins as acid amides. We may represent the structure by using glutamic acid as an example:

That all of the ammonia resulting from a 24 or 48 hour hydrolysis may not come from the amide nitrogen of the original protein molecule is illustrated by an experiment of Gortner and Holm (24). They found a definite value for ammonia nitrogen at the end of the first four hours of the hydrolysis, following which there was a small but steady increase in the amount over a period of six weeks when the experiment was discontinued. At this time a constant value had not yet been obtained. Other investigators have reported similar findings. Under the ordinary conditions of protein hydrolysis with acid, a certain amount of deamination probably takes place. There is the possibility, however, that these findings may be the result of the slow hydrolysis of some very stable amides. It has been shown that asparagine is more stable toward acid than the amide of glutamic acid. Only the monoamino acids are deaminized by boiling with acids. Arginine, histidine, and lysine, when treated similarly, yield no ammonia nitrogen within six weeks.

(7) Rate of Hydrolysis. When proteins are hydrolyzed by means of acid, alkali, or enzymes, the peptide linkages are broken. The rate of the hydrolysis, in the case of acids and alkalies, depends on the strength of the hydrolytic agent and the temperature, and, in the case of enzymes, on a variety of factors. Several methods for following the rate of hydrolysis of proteins have been proposed. Of these, the rate of liberation of amino and carboxyl groups, as determined by the Van Slyke amino nitrogen method and the Sörensen formol titration method, are probably the best indicators available.

The rates of hydrolysis by means of acids and alkalies have not been studied in a large number of cases, but in those which have been studied, it seems that alkalies are more rapid hydrolytic agents than acids of the same strength and that, in some instances at least, barium hydroxide acts somewhat more rapidly than does sodium hydroxide. There is very little difference between sulfuric acid and hydrochloric acid. The above statements are clearly illustrated in Figs. 3 and 4.

The rate and extent of hydrolysis by means of enzymes have been more extensively studied, but the conditions under which the experiments have been performed have precluded uniformity of results. In general, the hydrolysis is very rapid at the beginning of

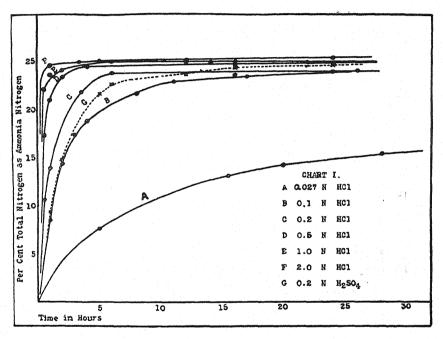


Fig. 3. Amide hydrolysis of gliadin by acids. (Vickery, H. B., J. Biol. Chem., 53, 495 (1922).)

the experiment, but slows down as the hydrolytic products accumulate and, after long periods of time, reaches an equilibrium. This equilibrium varies with temperature, pH, concentration of enzyme, and probably a number of other factors, but most important of all with the enzyme itself. The extent of hydrolysis of crystalline egg albumin by pepsin and the combination of pepsin and other enzymes is illustrated in Table I.

The rate of hydrolysis is not given in this table. However, it was found, when finely divided heat-coagulated egg albumin was treated with one tenth of its weight of pepsin at pH 2.5 and at 30°,

that 25 per cent of the total linkages which could be broken in 36 days, were broken within two hours. The final number of linkages split by pepsin was one third of the total number present in the molecule. Exactly analogous results have been found (25) for lactalbumin. The rate of hydrolysis of proteins by means of enzymes seems never to be so rapid as it is when acids and alkalies

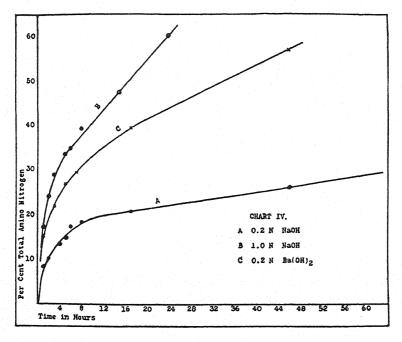


Fig. 4. Peptide hydrolysis of gliadin by alkalies. (Vickery, H. B., J. Biol. Chem., 53, 495 (1922).)

are used. The extent of hydrolysis may sometimes be, but seldom is, as great with a combination of enzymes as it is with a single acid or alkali.

3. SELECTIVE LIBERATION OF AMINO ACIDS

The rate at which amino acids are selectively liberated from proteins during hydrolysis with acids or alkalies has received very little study. Much more attention has been paid to the liberation of amino acids following hydrolysis by enzymes. Two general procedures have been employed, one of them being the actual isolation, and the other the determination of the amino acids by indirect methods. Tyrosine can be quite easily isolated if it is present;

Table II

Color Values Obtained by Folin and Marenzi's Method on Casein under Different Treatments

Experi- ment No.	Description	Color values
		per cent
1	Casein-pepsin mixture heated to 80°, tested at once	0.20
2	Casein-HCl mixture heated to 80°, cooled, tested at once	0.20
3	Casein-pepsin mixture tested at once without heating to 80°	0.19
4	Casein-HCl mixture tested at once without heating	0.18
5	Casein-pepsin mixture allowed to stand 24 hrs. at room tem-	0.21
	perature	
6	Casein-HCl mixture incubated 1 hr. at 35-38°	0.24
7	Casein suspended in distilled water 2 hrs. at room temperature. Tests made directly on suspension	0.24

(Jones, D. B., and Gersdorff, C. E. F., J. Biol. Chem., 101, 657 (1933).)

arginine can be determined by means of arginase and by isolation; and cystine and tryptophane can be determined colorimetrically. It is these four which have been most carefully followed. Only in those instances where a particular amino acid has actually been isolated do we have definite assurance that it is present in the free state in the hydrolysate. When present in the protein molecule, the amino acid may react with the same colorimetric reagents with which it reacts in the free state. In fact, the reactions may even be intensified.

Fig. 5 shows that the values obtained by the Folin and Marenzi method for the peptic digests and the acid hydrolysates of casein

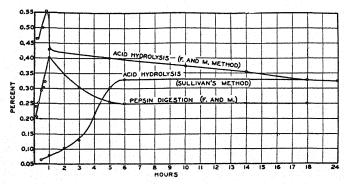


Fig. 5. Color values of hydrolysates and peptic digests of casein as determined by Folin and Marenzi's and Sullivan's methods for estimating cystine.

(Jones, D. B., and Gersdorff, C. E. F., J. Biol. Chem., 101, 657 (1933).)

follow the same general trend. There is first a rapid rise in values, followed by a very rapid drop, then a slow decrease until constant values are reached. These data are in striking contrast to those obtained by the Sullivan method which is quite specific for free cys-

Table III

Hydrolysis of Egg Albumin for Varying Lengths of Time

The amino nitrogen is expressed as percentage of the total nitrogen. All other values are expressed as percentages of ash- and moisture-free crystalline egg albumin.

		Chromogenic values calculated as									
Time	Amino N peptic		stine	Tyr	osine	Tryptophane					
	hydrolysis	Peptic	Acid hydrolysis	Peptic hydrolysis	Alkaline hydrolysis	Peptic hydrolysis	Alkaline hydrolysis				
hrs.											
0.25	3.5	0.78	0.37	0.50	2.0	0.72	0.00				
0.50	3.6	0.83	0.39	0.54	2.7	0.69	0.23				
0.75	4.2	0.99	0.55	0.65	2.9	0.68	0.25				
1.0	4.8	1.1	0.70	0.79	3.0	0.23	0.34				
1.5	5.3	1.2	0.72	0.95	3.4	0.23	0.46				
2.0	6.0	1.3	0.85	1.3	3.5	0.25	0.57				
3.0	6.5	1.6	0.97	1.4	3.6	0.26	0.73				
5.0	7.0	1.8	1.3	1.6	3.8	0.29	0.92				
8.0	7.6	2.0	1.3	1.8	4.6	0.39	1.2				
12.0	8.2	2.1	1.2	2.4	4.7	0.70	1.2				
24.0	9.3	2.0	0.72	2.6	4.7	0.78	1.3				
36.0	10	1.9	0.58	2.6	4.7	0.97	1.3				
days			1.5	La Teach							
4	13	1.8		2.7		1.0					
7	16	1.7		2.7		1.0					
14	20	1.7		2.7		1.1	1				
21	24	1.7		2.6		1.0					
36	25	1.6		2.6		1.0					

(Calvery, H. O., Block, W. D., and Schock, E. D., J. Biol. Chem., 113, 21, (1936).)

tine and which gave negative results in the case of the peptic digests. Cystine is not set free by peptic digestion of casein. The acid hydrolysates show continually increasing values until a maximum is reached which thereafter remain constant. The color values obtained by the Folin and Marenzi method on peptic hydrolysates are not due to splitting of protein by the hydrochloric acid (See Table II) which was present since, on treating the protein with hydrochloric acid under the same conditions, no such values were

obtained. The data do not represent free cystine. The test is given by some compound or compounds other than cystine, probably peptides of cystine or other reactive groups which are exposed in the partial cleavage products by pepsin. After reaching a maximum value they are rapidly converted to non-reactive compounds.

Table III illustrates a similar experiment with crystalline egg albumin. Chromogenic values for tyrosine, tryptophane, and cystine are recorded after peptic, acid, and alkaline hydrolysis. The values for cystine in egg albumin shown in the table differ quite markedly from those illustrated in Fig. 4 for casein. Prolonged acid hydrolysis gradually destroys cystine. In the case of peptic hydrolysis, the chromogenic values for cystine as determined by the Folin and Marenzi method are higher than those obtained by acid hydrolysis. There are at least four possible explanations for this: (a) there may be destruction of reactive groups by a process other than simple hydrolysis; (b) compounds containing -SH groups may be liberated which later are converted to -S-S- compounds; (c) peptides containing cystine which have a higher chromogenic value than cystine itself may be liberated. The peptides are later hydrolyzed by the acid and lose their high chromogenic value; (d) the method may be nonspecific or influenced by errors. Any one or all four of these explanations may be correct.

As a result of the early experiments of Abderhalden (26), it is almost universally stated that free amino acids are not liberated by the action of pepsin on protein. However, free lysine has been isolated from a peptic digest of histone (27), tyrosine has been isolated from a peptic autolysate (28), tyrosine has also been isolated from peptic digestion of crystalline egg albumin (29), and arginine has been isolated following peptic hydrolysis of several different proteins (30). With this amount of evidence it hardly seems possible to doubt any longer that some amino acids are liberated during peptic hydrolysis. They are not freed as a result of the acid medium in which pepsin acts.

"Tryptic" or pancreatic digestion of proteins can be sharply contrasted with peptic hydrolysis because of the much more rapid liberation of amino acids. Furthermore, a more accessible position or more readily hydrolyzable linkages enable trypsin to split off different amino acids at different rates, tyrosine and cystine appearing very soon, and glutamic acid more slowly (1, pp. 214–215). (See Table IV.)

The hydrolysis of gelatin by pepsin and trypsin and by acid and

alkali have been compared by Northrop (31), using the formol titration method to follow the rate and the extent of hydrolysis. He concludes that those linkages which are most easily hydrolyzed by pepsin are also more resistant to acid hydrolysis. Those which are broken by pepsin are also attacked by trypsin; but some of those which are broken by trypsin are resistant to pepsin. He further

Table IV

The Rate of Liberation of Tyrosine and Glutamic Acid in a

Pancreatic Digest of

(Results express percentage liberation of each amino acid.)

Casein						Edestin						
Days 1	3	6	9	11	13	17	21	1	2	3	7	16
Tyrosine16.7 Glutamic	97.8	96.9	96.5	93.3	100.0	99.1	96.9	78.4	97.6	97.6	100.0	100.0
acid14.2	16.3	45.2	83.4	80.1	81.5	82.4	85.5	4.3	7.4	10.9	31.1	60.2

(Mitchell, H. H., and Hamilton, T. S., The Biochemistry of the Amino Acids, New York, 1929, pp. 214 and 215.)

concludes that if certain bonds are broken by both enzymes, those which are rapidly hydrolyzed by pepsin are slowly broken by trypsin, and those which are attacked by pepsin and trypsin are most easily broken by alkali. These are justifiable conclusions from the data, and are not inconsistent with the conclusions of others.

Summarizing: proteins are hydrolyzed by the three groups of hydrolytic agents at different rates and to different extents; some amino acids are liberated almost completely very early by any one or all three of the hydrolytic agents, while some form very resistant groupings in the protein molecule. Tyrosine, cystine, and arginine are among those amino acids which are readily liberated, while proline and phenylalanine are among the resistant ones.

4. THE FISCHER ESTERIFICATION METHOD

Very little was known regarding the quantitative relationship of the monoamino acids in proteins until 1901 when Fischer (16) proposed the method which now bears his name. The principle of the method is the separation of the amino acids by the fractional distillation of their esters in vacuo. Fischer proposed it as a qualitative method, while subsequent investigators have modified it and used it with some success as a quantitative method. There are several quite complete descriptions and discussions of the details of the method (1, 2). The apparatus which is used is shown in Fig. 6. The principles involved and the more important procedures are:

(1) Hydrolysis. Approximately 300 grams of protein are hydrolyzed with hydrochloric or sulfuric acid. The mixture is cooled

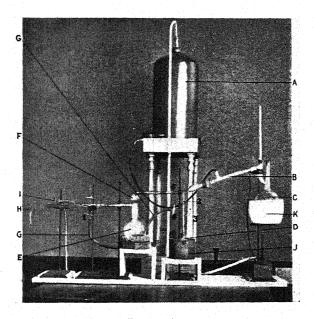


Fig. 6. Fischer Apparatus.

Reproduced from a photograph made by Prof. E. T. Reichert, of the University of Pennsylvania. The negative was furnished by Dr. T. B. Osborne, of New Haven, Conn.

A. Tank into which freezing mixture is pumped and from which it flows through the condenser, B; C, flask from which the esters are distilled, the distillate being collected in D; E, a Dewar flask containing liquid air serving as a cooler for condensing tube F: G and G', tubes leading to the Geryck pump by which the vacuum is maintained: I, tube leading to a McLeod gauge (not shown in figure); J, a bath containing freezing mixture in which the receiver D is immersed; K, a bath of water during the first part of the distillation and of oil during the last part of the process; 1–5, stop cocks which permit the cutting out of different parts of the apparatus as the procedure demands.

(Hawk, P. B., Practical Physiological Chemistry, 4th Ed., Philadelphia (1913).)

and filtered in order to remove humin. If sulfuric acid is used it is entirely removed, but if hydrochloric acid is used the removal of the acid is not necessary. Cystine and tyrosine may, in part, be separated from the other amino acids by crystallization at the proper pH.

- (2) Removal of Glutamic Acid Hydrochloride. The above solution is concentrated in vacuo to almost a thick syrup, then saturated at zero degrees with gaseous hydrogen chloride, and allowed to stand at this temperature for several days. The glutamic acid hydrochloride which crystallizes out is removed by filtration and washed. The solution is concentrated and again saturated with dry gaseous hydrogen chloride at 0° in order to remove as nearly as possible all of the glutamic acid as the hydrochloride.
- (3) The Esterification Process. The filtrate from the glutamic acid hydrochloride is freed from water by repeated evaporation with absolute ethyl alcohol. It is then saturated in absolute alcohol with gaseous hydrogen chloride and again evaporated to a syrup in order to remove the water resulting from esterification. Absolute alcohol is added and the solution is again saturated with dry gaseous hydrogen chloride. In this process stannous chloride is sometimes added. It may be necessary to repeat the above process a third time or more in order to be certain that complete esterification has taken place. There have been several modifications of the esterification process, some of which are preferred by some investigators. The esterification of the lead salts is a very excellent modification.
- (4) Glycine. On concentrating and cooling the mixture to 0° and allowing it to stand for a few days, any glycine which is present will precipitate as glycine ester hydrochloride. The precipitation is often facilitated by seeding with a crystal of the ester hydrochloride. Concentration of the mother liquors after filtration from the first crop of crystals may yield a second crop. The amino acids remaining in the mother liquor may then be reesterified if it is thought that any moisture may have caused hydrolysis of the esters during the process of isolation of the glycine ester hydrochoride.
- (5) The Removal of the Hydrochloric Acid. This is done by some appropriate technic, bearing in mind that the esters may be very readily hydrolyzed. An exact equivalent of sodium ethylate in absolute alcohol may be used. A strong solution of sodium hydroxide in a freezing mixture has been used. Barium hydroxide, as well as ammonia, or lead oxide, have been used to good advantage. After neutralization of the hydrochloric acid, the esters are extracted with ether or with ether and chloroform, the solution is dried, the solvents are removed, and the esters are distilled in vacuo.
 - (6) Fractional Distillation of the Esters. The ester fractions ob-

tained will depend upon the pressure under which the fractionation is made as well as the composition of the mixture of amino acids. A typical fractionation may be:

60° at 10 mm. (the esters of glycine, alanine, leucine, and proline.)

60° to 100° at 10 mm. (the esters of valine, leucine, and proline.)

100° to 130° at 0.5 mm. (the esters of leucine and proline).

130° to 180° at 0.5 mm. (the esters of phenylalanine, glutamic acid, aspartic acid, and serine).

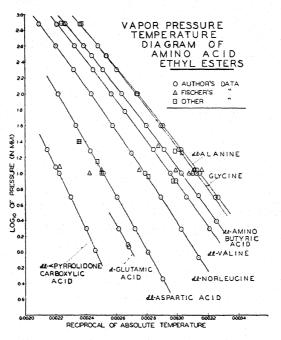


Fig. 7. Vapor pressure diagram of amino acid ethyl esters.

(Dunn, M. S., Smart, B. W., Redemann, C. E., and Smith, N. L., (personal communication).)

There is considerable overlapping in this procedure with no fraction consisting of a single amino acid ester. The residue which does not distill contains the esters of the basic amino acids, cystine, and others. Recent vapor pressure measurements of amino acid esters are shown in Fig. 7.

(7) The Amino Acids. The separation of the individual amino acids of the various fractions is different for each amino acid, thus

making a rather long and difficult process before the analysis is complete. The loss, even when pure amino acids are used as the starting material, is about 30 per cent. This is illustrated in Table V which is reproduced from Osborne and Jones (32).

Table V
Summation of the Analysis of the Mixture of Pure Amino Acids

		Recove	red from		Recovered from			
Amino acids taken		Esters I	Esters II	Total	Esters I	Esters II	Total	
	gm.				per cent			
Alanine	16.00	7.34	0.00	7.34	45.88	0.00	45.88	
Valine	4.00	1.40	0.24	1.64	35.00	6.00	41.00	
Leucine	85.00	53.18	16.18	69.36	62.56	19.04	81.60	
Proline	31.00	19.54	3.02	22.56	63.03	9.74	72.77	
Phenylalanine	26.00	15.64	2.43	18.07	60.16	9.35	69.51	
Aspartic acid	6.00	2.55	0.00	2.55	42.50	0.00	42.50	
Glutamic acid	120.20	50.11	33.15	83.26	41.76	27.63	69.39	
Tyrosine	16.00	7.56	0.41	7.97	47.25	2.56	49.81	
Arginine	5.50	3.57	0.00	3.57	64	.91	64.91	
Histidine	2.04	0.80	0.00	0.80	39	.21	39.21	
Serine	2.00	0.00	0.00	0.00	0	.00	0.00	
Ammonia	14.31							
Total	328.05	161.69	55.43	217.12			66.17	

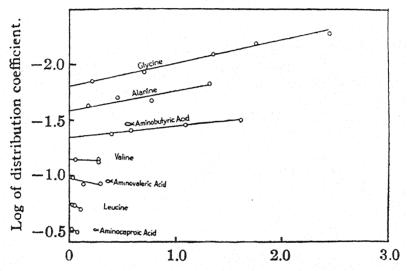
(Osborne, T. B., and Jones, D. B., Amer. J. Physiol., 26, 305 (1910).)

It is obvious from this table that the method is far from quantitative. Better recovery could certainly not be expected from a protein hydrolysate than from a mixture of pure amino acids. The following sources of error may be mentioned: (a) incomplete hydrolysis, (b) loss through formation of humin, (c) incomplete esterification, (d) decomposition of esters by hydrolysis prior to distillation, (e) unavoidable loss in the fractional crystallization of the amino acids, (f) conversion of esters into diketopiperazines, and (g) decomposition during the distillation process.

5. DAKIN'S BUTYL ALCOHOL METHOD

The stimulus which led Dakin to the development of this method was a desire to obtain some optically pure proline. As the ester method invariably gave a partially racemized product, attempts were made to extract proline from a hydrolysate of casein by means

of various alcohols. Dakin studied gelatin, casein, and zein (33). Other proteins have since been investigated. The method depends on the distribution of the amino acids between two phases: water saturated with butyl alcohol, and butyl alcohol saturated with water. The distribution coefficients of some of the amino acids between these phases have been determined by England and Cohn (34). Their data are represented in Figs. 8, 9 and 10.



Amino acid in water phase, moles per liter.

Fig. 8. Distribution coefficients of amino acids between butyl alcohol and water. (England, A. Jr., and Cohn, E. J., J. Amer. Chem. Soc., 57, 634 (1935).)

The general procedure in the Dakin technique is as follows: the protein is hydrolyzed with sulfuric acid and the acid is removed by barium hydroxide. The resulting solution is concentrated to a thin syrup and extracted in a continuous extractor in vacuo with butyl alcohol (see Fig. 11). Large quantities of solid amino acids separate from the alcoholic extract. Proline and practically all of the monoaminomonocarboxylic acids are extracted, together with peptide anhydrides if they are present. Only traces of the strongly ionized dibasic and dicarboxylic amino acids are extracted, the major portion being left in the aqueous solution. Of the monoaminomonocarboxylic acids, glycine is very difficult to extract, and serine is also more slowly extracted. Hydroxyproline is more readily extracted than glycine when the extraction is carried out at ordinary atmospheric pressures. Very little glycine is extracted by

butyl alcohol when the extraction is carried out at 10 mm. pressure. The extraction of 300 grams of mixed amino acids may be complete in 36 hours.

The solid cream-colored amino acids which separate from the butyl alcohol are filtered off, washed with a little butyl alcohol, and then with ether which removes most of the pigment. The mother liquor contains the whole of the proline, together with small amounts of other amino acids and anhydrides. They can be sepa-

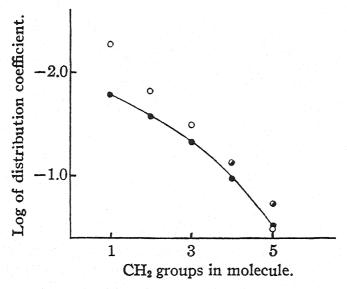


Fig. 9. ○, n-Amino acids in saturated solution; ●, n-amino acids at infinite dilution; half shade circle, branched amino acids at infinite dilution.

(England, A. Jr., and Cohn, E. J., J. Amer. Chem. Soc., 57, 634 (1935).)

rated from each other by taking advantage of their relative solubilities in alcohol and water.

The residue of non-extracted amino acids contains practically all of the basic amino acids and also the dicarboxylic amino acids, from which both groups of amino acids may be obtained in excellent yields. As pointed out above, when the extraction is continued for only 36 hours, only traces of these two groups of amino acids are found in the butyl alcohol extract. Thus, by the use of butyl alcohol, the products of hydrolysis of a protein may be readily separated into the following five groups:

1. Monoaminomonocarboxylic acids, both aliphatic and aromatic, insoluble in ethyl alcohol, but extracted with butyl alcohol.

- 2. Proline, soluble in absolute ethyl alcohol and extracted with butyl alcohol. Hydroxyproline is extracted at atmospheric pressures, but not when the extraction is carried out at 10 mm. Propyl alcohol may be used to extract hydroxyproline under reduced pressure.
- 3. Peptide anhydrides (diketopiperazines) extracted by butyl alcohol, but separated from group 2 by sparing solubility in alcohol or water.

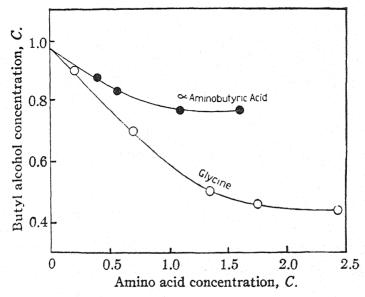


Fig. 10. Influence of amino acids upon solubility of butyl alcohol in water. (England, A. Jr., and Cohn, E. J., J. Amer. Chem. Soc., 57, 634 (1935).)

- 4. Dicarboxylic acids, not extracted by butyl alcohol.
- 5. Diamino acids, not extracted by butyl alcohol, but separable from group 4 by phosphotungstic acid or by other means.

The following points should be appreciated by those who wish to use the method. (a) The groups are composed of chemically similar individuals. (b) Practically the whole of the products of the hydrolysis of a protein may be divided into the five groups without serious loss, and each can readily be obtained in solid form, aliquot parts of which may be used for the isolation of individual amino acids. (c) There is no indication that racemization occurs. All of the amino acids so far investigated possess their full optical activity. The absence of racemization is of obvious impor-

tance when the method is used to prepare the material for bacterial or animal metabolism experiments. (d) High yields of several of the amino acids may be obtained by this method, thus permitting a more nearly quantitative analysis of the proteins. (e) If large quantities of glycine are present in the protein which is under investigation, difficulties can be anticipated since this amino acid is only very slowly extracted and seemingly never quantitatively.

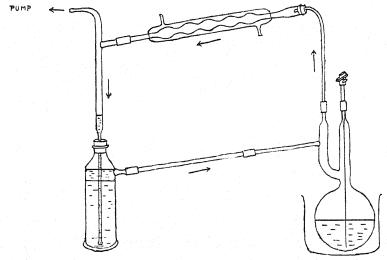


Fig. 11. Apparatus for extracting amino acids with butyl alcohol.
(Dakin, H. D., J. Biol. Chem., 44, 499 (1920).)
(See also, Fayolle et Lormand, C., Chimie et Industrie, 8, 273 (1922).)

(f) When the extraction is prolonged, large quantities of glutamic acid and appreciable quantities of the hexone bases will be extracted. The use of this method led Dakin to the discovery of β -hydroxyglutamic acid. A modification of the method in which the amino acids were not heated so long has been used with some success, but it is not quite so convenient. (g) Tryptophane can be extracted almost quantitatively in a very short time from a tryptic hydrolysate of casein. One purification with mercuric sulfate is sufficient in order to obtain this amino acid in a fair state of purity. It should be emphasized that the amounts of certain amino acids which are extracted by butyl alcohol depend on the pressure under which the extraction is carried out.

6. THE ELECTRICAL TRANSPORT METHOD

Electrical transport was first used for the separation of glutamic acid from a protein hydrolysate. The method was patented in 1912 (35). The monosodium salt of glutamic acid (Ajinomoto, Kin-saji, etc.) is extensively used in the Orient as a condiment. Electrical transport has not as yet been used as a quantitative procedure for the determination of amino acids, but it possibly should be given more consideration in this connection. The method is very useful for the isolation of certain amino acids (36). The procedure is based on the influence of pH on the dissociation of the amino

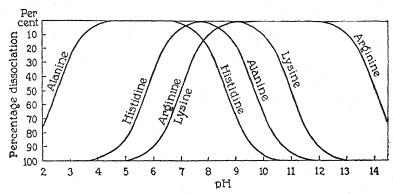


Fig. 12. Percentage dissociation of amino acids at varying pH values. (Foster, G. L., and Schmidt, C. L. A., J. Biol. Chem., 56, 545 (1923).)

acids (see Fig. 12). On placing a protein hydrolysate, which is maintained at pH 5.5, in the middle compartment of a three-compartment cell, and passing a direct current through the solution, the amino acids are soon found to separate rather sharply into three fractions: (a) the amino acids which are predominantly acidic, viz., aspartic, glutamic, and β -hydroxyglutamic acid, which migrate toward the anode; (b) the basic amino acids, arginine, histidine, and lysine, which migrate toward the cathode; (c) the monoamino monocarboxylic acids, which are not appreciably dissociated at this pH, remain in the center compartment. On adjusting the pH of the solution of the basic amino acids to pH 7.5, and again using electrical transport, arginine and lysine migrate to the cathode, while histidine remains in the center compartment. The method has been used (37) for preparation of large quantities of histidine from a hydrolysate of blood corpuscle paste. The apparatus used by Schmidt and Foster is illustrated in Figure 13.

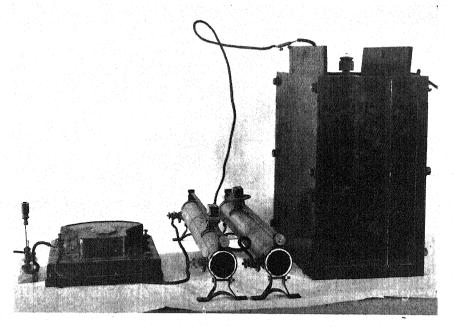


Fig. 13. Backelite cell used by Schmidt and Foster for the separation of amino acids by electrical transport.

7. SPECIAL ACIDS USED FOR THE PREPARATION OF AMINO ACIDS

(1) Flavianic Acid.

In his early investigations of the sperm cells of fish, Kossel found that naphthol yellow-S, the sodium salt of 1-naphthol-2, 4-dinitro-7-sulfonic acid (flavianic acid), stained the nuclei of these cells. Years later he (7) returned to this early observation and found that flavianic acid forms definite crystalline compounds with certain organic bases. The observation was applied to arginine with the result that a highly insoluble, beautifully crystalline compound, which contained two molecules of flavianic acid and one of arginine, was obtained. It is the most insoluble compound of arginine known. Use has been made of this brilliant observation in the preparation of arginine in large quantities directly from protein hydrolysates

(38). The compound is so insoluble that, unlike the compounds formed by many precipitating agents, it will crystallize almost quantitatively even in the presence of all of the other amino acids. Histidine forms both a mono- and a diflavianate salt, while flavianates of lysine and ornithine have thus far not been isolated. In Chapter IV the quantitative determination of arginine and histidine by means of flavianic acid as a final step in the Kossel and Kutscher silver method is discussed in detail.

(2) Picric acid.

$$O_2N$$
 NO_2
 NO_2

When a saturated solution of picric acid or the solid itself is stirred with a protein solution, the protein will usually be precipitated practically quantitatively from the solution as a picrate. Sometimes adjustment of pH is necessary. In the compounds formed, apparently the picric acid (and likewise some other orthonitrophenolic compounds) and the protein form definite reproducible compounds which can be used as a quantitative measure of the protein. When two grams of hemoglobin, casein, or edestin are treated with 100 cc. of a 3 per cent alcoholic solution of picric acid on a water bath for one hour with constant stirring, a yellow compound is formed, the depth of the color depending on the phenolic compound used. When the solution is cooled, filtered, and the precipitate is washed with alcohol, ether, and benzene, and dried in vacuo, the phenolic content can be determined by titration with methylene blue. This gives a quantitative measure of the amount of protein used.

Several of the amino acids, when mixed in concentrated solution with about four molecular equivalents of picric acid dissolved in alcohol, form definite crystalline compounds which can be used for identification and characterization. The picrates of glycine, proline, arginine, histidine, and lysine are well characterized products (See Chapter IX, section on crystallization).

In the Abderhalden test (39) for diketopiperazines, the protein is treated with an alkaline solution of pieric acid. A reddish brown color is obtained. Meta-dinitrobenzene and 1,3,5-dinitrobenzoic

acid may also be used to test for diketopiperazines. A large number of proteins, including silk, keratin, ricin, and gelatin, give a positive test. The fact that substances other than diketopiperazines give positive tests with these reagents causes some doubt regarding Abderhalden's interpretation of the reaction.

(3) Picrolonic Acid.

$$NO_2$$
 OH
 $C = C - NO$
 $N = C - CH_3$

Like flavianic acid, picrolonic acid was first used to characterize the basic amino acids. All of the natural monoaminomonocarboxylic acids form definite salts, with the exception of proline and hydroxyproline. Most of the compounds are quite insoluble in water. The picrolonates of phenylalanine and tryptophane are particularly insoluble in water. The amino acids are quite easily recovered in quantitative amounts from the picrolonate by addition of acid and extraction of picrolonic acid with ether.

(4) Reinecke Salt. [(NH₃)₂Cr(CNS)₄·]NH₄ (Ammonium tetrathiocyano-diaminochromite). This compound was first used in protein studies for the preparation of pure proline and hydroxyproline (40). It has occasionally been used for some of the other amino acids, but has not found wide application. Recently Bergmann has substituted aniline for ammonia in Reinecke salt and obtained a compound which he calls "rhodanilic acid." This compound serves as an excellent means for the determination of proline. The guanidine salt has likewise been used. It has been designated as "Morland salt."

Several other organic acids and acid chlorides have been used to form acid derivatives of both proteins and amino acids. Both phenylisocyanic acid and α -naphtholisocyanic acid in alkaline solution form such derivatives. For example, one of the recent uses of phenylisocyanate was to demonstrate that at least a part of the phenylalanine of insulin is attached in such a manner that the amino group is free. Presumably the phenylalanine is attached through the carboxyl group at the end of the chain. When insulin is treated with phenylisocyanate in alkaline solution, a precipitate

is formed which, when filtered, washed, and hydrolyzed with strong hydrochloric acid, yields the phenylhydantoin of phenylalanine (41). The phenylisocyanate derivative is converted immediately by the strong hydrochloric acid into the hydantoin.

Four acid chlorides that have been extensively used for the formation of derivatives of proteins, and especially of the amino acids, are benzoyl chloride, β -naphtholsulfonyl chloride, 4-nitrotoluol-2-sulfonyl chloride, and benzenesulfonyl chloride. The general procedure for the preparation of these derivatives is the following: an aqueous solution of the amino acid is made slightly alkaline with sodium hydroxide; a slight excess over a molecular quantity of the acid chloride and sodium hydroxide are then added alternately in small portions. The mixture is first shaken in an ice bath, then at room temperature until the odor of the acid chloride disappears. The solution is acidified and, after cooling, the acids are filtered off and separated by differences in solubility in suitable solvents.

The list would not be complete unless mention was made of the acid chloride, carbobenzoxy chloride C₆H₅—CH₂O—CO—Cl, which has been used so successfully by Bergmann and his co-workers for the synthesis of a large number of new peptides which were not obtainable by previous methods (See Chapter VI).

(5) Heavy Metals. When protein solutions are treated with salts of certain heavy metals, precipitates of metallic proteinates are formed. Under similar conditions several of the amino acids likewise form salts. In many cases these are definite crystalline compounds. All of the metallic compounds which have been used for the quantitative removal of protein from solution or for the quantitative separation of the individual amino acids need not be mentioned here. Those that are most often used are mercuric chloride, mercuric sulfate, mercuric nitrate, potassium mercuric chloride, potassium-zinc iodide, bismuth nitrate, gold chloride, platinic chloride, silver nitrate, cupric chloride, cupric sulfate, cupric oxide, mercuric acetate, zinc oxide, zinc chloride, zinc acetate, lead nitrate, lead chloride, phosphotungstic acid, and phosphomolybdic acid.

In many cases when the amino acids react with the neutral salts of the heavy metals, crystalline double salts of definite composition are formed that may be used for purposes of identification. Probably the best of these reagents is mercuric acetate which, in the presence of carbonates, has been used for the quantitative precipi-

tation of the amino acids. The salts most commonly used for the isolation of the amino acids are those of silver, mercury, lead, zinc, copper, barium, and calcium. The reaction may be illustrated with mercuric chloride:

Several special applications of the use of the salts of heavy metals for the isolation of amino acids, in many cases in quantitative amounts, from protein hydrolysates have been made. The use of phosphotungstic acid for the quantitative separation of the basic amino acids and cystine in the Van Slyke distribution method is discussed in detail in Chapter IV. For the precipitation of cystine and tryptophane, a mercuric sulfate solution in 5 per cent sulfuric acid has been used quite often since the mercuric compound of tyrosine is soluble in 5 per cent sulfuric acid. The method is not entirely quantitative for cystine. Some tyrosine is lost. There is a modification of this method for cystine. Subsequent to precipitation of the cystine with mercuric sulfate, the mercury is removed with hydrogen sulfide and the cystine is precipitated by means of copper hydroxide. The small amount of tyrosine present as a contaminant forms a soluble copper salt and remains in solution. The yield of highly purified cystine obtained by this method is much higher than have previously been obtained from the same proteins by the use of other reagents.

Tryptophane was first isolated from protein by means of mercuric sulfate. On treating a tryptic digest of protein with a solution of mercuric sulfate in 5 per cent sulfuric acid, cystine, tryptophane, and small amounts of tyrosine are precipitated. On washing the precipitate with 5 per cent sulfuric acid, the tyrosine salt is dissolved and the salts of tryptophane and cystine remain. The precipitate is decomposed with hydrogen sulfide and the solution is fractionally precipitated with mercuric sulfate, the cystine precipitating first and finally the tryptophane. The mercury is removed from the tryptophane fraction by means of hydrogen sulfide and the amino acid is recrystallized from water. The method is now modified as mentioned earlier by separating the tryptophane from the cystine by extraction with butyl alcohol.

Another amino acid which can be precipitated practically quantitatively with the above reagent, especially when the solution is

concentrated and only small amounts of other amino acids are present, is histidine. The concentration of the sulfuric acid must not be above 5 per cent by weight as is illustrated by the data in Table VI. This fact has often been made use of in several of the quantitative isolation methods for histidine.

TABLE VI

Standard Solutions of Pure Histidine in Sulfuric Acid Treated with an Excess of Hopkins' Reagent, and Various Dilutions Made

The concentration of sulfuric acid was estimated accurately by titration. The amount of material recovered was estimated as nitrogen and expressed in terms of total nitrogen present in the original solution. The experiments were continued for 48 hours.

Experi- ment No.	Histidine solution	Hopkins' reagent	Water added	Concentra- tion of sulfuric acid by weight	N recovered	Nitrogen recovered after second dilution
	cc.	cc.	cc.	per cent	per cent of total N	per cent
1	10	10	None	17.12	None	92.31
2	20	15	10	13.36	"	91.38
3	10	7	10	13.08	u	93.01
4	25	25	None	10.17	9.55	92.82
5	25	25	5	9.60	32.26	93.91
6	25	25	15	8.30	45.23	92.87
7	10	10	18	5.72	91.33	93.76
8	10	10	25	4.20	93.80	94.22
9	10	10	25	4.26	93.60	94.03

(Calvery, H. O., J. Biol. Chem., 83, 644 (1929).)

Silver has been used from time to time by various workers for the separation and characterization of several of the amino acids. Its use in the quantitative separation and determination of arginine and histidine is discussed in detail in Chapter IV under Kossel's silver method. Silver was also used as early as 1903 for the isolation of glutamic and aspartic acid. In this procedure the basic amino acids as well as chloride ions were removed with silver. The silver salts of glutamic and aspartic acids were then precipitated by the addition of an excess of a soluble silver salt and careful further addition of barium hydroxide. They were separated from each other, after removal of the silver as sulfide by precipitation of the glutamic acid as the hydrochloride or as the more insoluble zinc salt. The zinc salt of aspartic acid being much more soluble, remains in solution. The use of zinc is further discussed in the method of Brazier.

Another instance where silver proved of remarkable value was in the isolation of β -hydroxyglutamic acid from casein by Dakin (33). The monoaminomonocarboxylic acids were extracted with butyl alcohol, glutamic acid was separated as the hydrochloride, and aspartic acid as the lead salt. After precipitation of the lead from the filtrate as lead sulfide, the basic amino acids were precipitated as the phosphotungstates. The phosphotungstic acid was then removed as the barium salt and the last traces of chlorides were removed by addition of nitric acid and silver nitrate. The solution was made faintly alkaline and the new amino acid, β -hydroxyglutamic acid, was precipitated as a white silver salt by alternate addition of silver nitrate and sodium hydroxide until silver ions were present in excess, as shown by the formation of brown silver oxide.

Glutamic and aspartic acids have been separated from the other amino acids of a protein hydrolysate as the calcium or barium salts since they are insoluble in alcohol. In the Foreman (129) procedure the hydrolysate is evaporated to a syrup, the syrup is dissolved in water, and an aqueous suspension of calcium hydroxide is added. The excess of lime and humin are filtered off and the filtrate is concentrated to remove ammonia. The solution of amino acids is poured into a large volume of alcohol. The calcium salts of aspartic and glutamic acids precipitate. The calcium is removed as oxalate, the excess of oxalic acid is precipitated with silver, and the excess of silver is removed as silver sulfide. The glutamic and aspartic acids are then separated in the usual manner. A modification of this method (42), in which barium is substituted for calcium, is now used almost exclusively. The barium is much more easily removed, than the calcium, and the procedure is shortened considerably.

A method in which copper and zinc salts are the principal reagents used is that of Schryver as modified and extended by Brazier (43). Schryver's object was to find a method for the separation of the hydrolytic products of protein that, as far as possible, should fulfill the following conditions:

- (a) the method should be capable of furnishing a maximum yield of the individual amino acids;
- (b) the use of the ester method should be avoided entirely;
- (c) the method should be as simple as possible.

The technique evolved, though not perfect, makes use of the fact that the different amino acids and certain of their derivatives, in particular their copper salts, show widely different degrees of solubility in solvents such as water, methyl alcohol, and ethyl alcohol. The success of the method depends upon the use of very pure dry solvents and thoroughly dry copper salts. By the use of absolute acetone, the copper salts are obtained in the form of a fine dry powder, which readily responds to fractionation by means of the appropriate solvents. The principal fractions are the following:

I. Copper salts insoluble in water.

This fraction contains leucine, phenylalanine, and aspartic acid.

II. Copper salts soluble in water.

These are further separated by methyl alcohol into the groups:

- (a) Copper salts which are insoluble in methyl alcohol: alanine, tyrosine, glycine, lysine, arginine, histidine, and glutamic acid.
- (b) Copper salts which are soluble in methyl alcohol: valine, hydroxyvaline, proline, and prolylphenylalanine.

The further separation of the individual amino acids of fraction I involves separation of aspartic acid as the barium salt and finally as the free acid, the removal of most of the leucine by fractional crystallization of the free amino acid mixture, and, finally, the fractional crystallization of the zinc salts of phenylalanine and the remaining leucine. The zinc salts are decomposed with hydrogen sulfide in alkaline solution and the free amino acids are crystallized. From fraction II (a) glutamic acid is removed as the barium salt, tyrosine is fractionally crystallized, histidine is separated as the zinc salt, arginine is subsequently removed as the flavianate, and, finally, alanine is removed by extraction with butyl alcohol.

The copper is precipitated from fraction II (b) by treating with hydrogen sulfide and any material which will crystallize is permitted to do so. The filtrate is then concentrated to a syrup, dried with acetone, and extracted with absolute alcohol. Proline is thus removed. The amino acids which are insoluble in alcohol are redissolved in water and from this solution hydroxyvaline, if present, may be isolated as the zinc salt. The method was applied to zein by Brazier. The results are shown in Table VII. As a general

method it is not very applicable, but certain parts of the procedure may prove very valuable.

 $\begin{tabular}{ll} Table VII \\ Total nitrogen to be accounted for = $35.33 gm. \\ \end{tabular}$

		N isolated	N as percentage
	Amino-acid	gm.	of total N
	Aspartic acid	0.68	1.92
Α.	{Leucine	5.31	15.05
	Phenylalanine	1.28	3.62
	Glutamic acid	6.78	19.19
	Tyrosine	0.39	1.14
В 1.	{Histidine		1.98
	Arginine	0.31	0.88
	(Alanine	1.68	4.76
((Prolylphenylalanine	0.35	0.99
(i)	{Proline		6.20
B 2. {	Alcohol-soluble fraction	2.42	6.85
(::)	Valine	1.01	2.86
(ii)	{Hydroxyvaline	. 0.53	1.50
C.	Ammonia	. 7.40	20.95
	Total	31.03	87.89

The total shows 87.89% of the nitrogen as amino acids isolated in an approximately pure form; 5.6% more of the nitrogen was found in precipitates, 93.5% in all being traced.

(Brazier, M. A. B., Biochem. J., 24, 1188 (1930).)

A quantitative method for the estimation of cystine based on the formation of the copper salt of cysteine has been proposed (44). The protein is hydrolyzed with sulfuric acid in the presence of metallic tin. The tin reduces cystine to cysteine. When an excess of cuprous oxide is added, the cysteine is quantitatively precipitated. The copper is then removed with hydrogen sulfide in the presence of a small amount of hydrochloric acid. The pH of the solution is subsequently adjusted to 7.2 and aerated to reconvert the cysteine to cystine. The solution is then acidified with hydrochloric acid and the sulfate ion is removed as the barium salt. The cystine content of the solution is calculated from the total nitrogen and the total sulfur content. The two values usually agree quite closely. When cystine was added to protein hydrolysates, 93 to 95 per cent could be recovered and, when pure cystine was used, the recovery was 98 to 99 per cent. The determination of cystine in a number of protein hydrolysates gave values which checked quite

closely with those obtained by the colorimetric method of Folin and Marenzi. The method can perhaps be used to check other methods used in estimating cystine. A disadvantage in this procedure is that, if other sulfhydryl compounds are present, they are precipitated along with the cystine and consequently the method is not specific. The method has recently been improved by Rossouw and Wilken-Jorden (45).

8. THE RACEMIZATION OF PROTEINS AND AMINO ACIDS

With the exception of glycine, all of the amino acids which have been obtained from natural sources are optically active since each contains at least one optically active carbon atom. Cystine, hydroxyproline, β -hydroxyglutamic acid, threonine, and isoleucine possess two optically active carbon atoms, hence four stereoisomers are possible. In certain procedures in the laboratory the optical form of the amino acid may in part be converted into its isomer and, when equal quantities of the two isomers are present in solution, a racemic mixture is the result. In such case no optical activity can be demonstrated. The conversion of an optical isomer into equal amounts of its antipodes is the process of racemization.

Racemization may be brought about by four methods:

(1) Proteins and polypeptides may be easily racemized by treatment with alkali. The free amino acids are not so easily racemized by this procedure. When a protein is dissolved in dilute alkali there is a gradual fall in the specific optical rotation to a value which is much lower than the initial value. It finally becomes constant. Similar changes occur with the hydantoins when they are allowed to stand in dilute alkali. The amino acids obtained from these hydantoins are optically inactive. Dakin explains this phenomenon (46) by the following series of equations.

¹ The first two steps in this series of reactions are carried out by appropriate synthetic methods.

While working with certain proteins, for example, gelatin, egg albumin, and casein, which had previously been treated with alkali in order to racemize them, Dakin and his co-workers observed, on hydrolysis of the protein, that some of the amino acids were racemized while others were obtained in an optically active form. Gelatin yielded inactive leucine, aspartic acid, arginine, histidine, phenylalanine, and, in part, alanine. Proline, glutamic acid, and lysine were isolated in the optically active form. Failure of some of the amino acids to racemize is attributed to the position of the amino acids in the protein molecule. Those having a free carboxyl group are not racemized. Only those amino acids which have the carboxyl group bound show enolization of the —CH—CO—group

to $-\dot{C} = \dot{C}$ —OH. This rearrangement is apparently necessary in order that racemization of the amino acids in a protein hydrolysate can take place. This accounts for the racemization of one amino acid in a dipeptide, two in a tripeptide, etc., as well as the failure of free amino acids to racemize.

Since the work of Dakin and his co-workers on the racemization of amino acids in several of the naturally occurring proteins, Levene and his co-workers (47) have made an extensive study of the action of alkali of varying concentrations on proteins, polypeptides, piperazines, and amino acids. They found that the free amino acids, as previously noted, were not racemized, while all three of the other groups of compounds were racemized even in very dilute alkali. They added 0.2 N, 1 N, and 5 N sodium hydroxide to albumin, casein, fibrin, gelatin, and edestin and found that each of these proteins was racemized by each of the alkalies. Polypeptides and piperazines were likewise racemized. In the case of the piperazines it was noted that they were also rapidly hydrolyzed. In discussing the results Levene has stated, "Thus the experimental results are as expected on the assumption that the racemization of the amino acids in the ketopiperazines is brought about by a tautomeric change (enolization) involving the asymmetric carbon atom." This is illustrated by the formulas:

(2) Racemization by means of heat in strong acid solution. Inactive cystine was first prepared by Hoffman and Gortner (48). The l-cystine was refluxed with strong hydrochloric acid on a sand bath for a long period of time. Optically inactive cystine was isolated from this solution. Whether the resulting product was composed of a racemic mixture of d- and l-cystine or whether it was the internally compensated mesocystine was not determined by these investigators, and no positive evidence was brought forth demonstrating the presence of either the racemic or the meso form until later. In order to prove the presence of either or both of these forms in the solution, it was necessary to isolate the different forms. The presence of the racemic form was demonstrated by the isolation of d-cystine from such an inactive mixture as mentioned above by Hollander and du Vigneaud (49). The proof of the presence of mesocystine was somewhat more difficult. It was necessary to actually isolate the mesocystine and to demonstrate its failure to resolve under conditions exactly the same as those that would bring about the resolution of racemic cystine. This has finally been accomplished and we now have l-cystine, d-cystine, mesocystine, and racemic cystine, that is, a mixture of equal quantities of d- and l-cystine (50).

It should be mentioned in this connection that racemization of cystine occurs when it is exposed to light for a long period of time in the presence of strong acid (51).

(3) Racemization by means of acetic anhydride. Another method for the racemization of amino acids was discovered by Bergmann and Zervas (130) who attempted to acetylate amino acids with acetic anhydride and found that racemization took place during this process. They have stated that in the acetylation of amino acids three phases are to be distinguished. In the first phase, when not more than one mole of acetic anhydride is used, the amino group is acetylated with retention of full optical activity. In the second phase, on further reaction with a mole of acetic anhydride, the amino acid becomes completely racemized but not altered in composition. With much larger amounts of the acetic anhydride, that is, several moles, the acetylated racemized amino acid loses water. The second phase proceeds with unusual velocity, the acetic anhydride acting catalytically. This same type of catalysis can be produced with carbon dioxide and benzoic acid anhydride. A possible explanation of this type of rearrangement is illustrated by the reactions:

(4) Racemization by acetic anhydride and sodium hydroxide. A method of racemizing amino acids which gives promise of being of general use was discovered when du Vigneaud and Sealock (52) attempted to prepare acetyltryptophane. In view of the difficulty of racemizing amino acids, the advantages of such a method are at once apparent. The study of the structure of amino acids would be greatly assisted since the racemic synthetic products which are always obtained in the synthesis of amino acids could be immediately compared, without resolution, with the racemized naturally occurring amino acids. It would also be of assistance when the isomer not occurring in nature is desired. The conditions under which these workers carried out their reaction differ slightly from the procedure used by other investigators for the acetylation of tryptophane. The product obtained by them also differs in crystalline structure and in melting point. The studies of Bergmann and Zervas on the catalytic racemization of amino acids by acetic anhydride suggested the possibility that racemization might have occurred in the usual method of acetylation of tryptophane. This would explain the difference in the two acetylation products. This was found to be the case since the acetyltryptophane prepared without an excess of acetic anhydride was not racemized, while that prepared and permitted to remain at 30° to 40° for 2 or 3 hours with an excess of acetic anhydride was completely racemized.

In order to show that the method is generally applicable to amino acids, the following were selected for investigation: methionine and cystine, representing the sulfur-containing amino acids; glutamic acid, representing the dicarboxylic acids; arginine, as an example of the basic amino acids; tyrosine and phenylalanine, as illustrative of the monoaminomonocarboxylic acids; and finally, proline, which contains an imino nitrogen. The sodium salts of the acetyl derivatives of all of these amino acids, with the exception of proline, were racemized in aqueous solution by acetic anhydride. In

the case of proline, no racemization occurred, and in the case of cystine, decomposition occurred simultaneously. The ease of racemization differed somewhat with the various amino acids since, in the interval of time in which acetyltryptophane is completely racemized, acetylglutamic acid is only partially racemized.

The mechanism of racemization of the sodium salt of amino acids in acetic anhydride is illustrated by the series of reactions given below:

Each of the above methods of racemization of amino acids has its advantages and disadvantages. For example, methods (1), (3), and (4) cannot be used for cystine, while method (2) can readily be used for cystine. The first method is particularly adaptable to the racemization of proteins and polypeptides, the fourth cannot be used for the racemization of proline and cystine. The last method seems to be the one which is most generally applicable.

9. THE RESOLUTION OF AMINO ACIDS

The process of resolution of amino acids consists in the separation of a racemic mixture into its optical isomers. As illustrated above, racemization may occur under various conditions and especially in the synthesis of amino acids. With the exception of glycine, the naturally occurring amino acids are optically active. In many organic compounds there is a marked difference in the physiological action between the d- and the l-forms and this difference holds particularly in the case of the biological use of the two different forms of the amino acids. In some cases the one form cannot be utilized by the organism, whereas the other, the naturally occurring one, may be essential for some biological process. For many purposes it is necessary to resolve the synthetic amino acid into the optically active forms.

There are three general methods available for the resolution of a

racemic mixture of amino acids into its optically active components. The first of these is the method of mechanical separation, illustrated by the classical experiment of Pasteur who used it to separate d- and l-tartaric acids. When the process of crystallization is slow, large crystals may be formed and, since the crystals of the d- and l-forms are mirror images of each other, it is possible to pick them out by mechanical means. It is rarely ever possible to use this method for the separation of amino acids or their derivatives since they seldom crystallize large enough for mechanical separation.

The second method of separation of the optical forms of the amino acids is a biological one. Many organisms show a surprisingly high degree of specificity for organic compounds. Yeasts, molds, and bacteria as well as some of the higher forms of animals, show a selectivity for only one form of the active amino acid. In general, only the optical form occurring in nature is the one which can be attacked by living organisms. In the biological method the synthetic amino acid is inoculated with a pure culture of bacteria, yeast, or fungus, and the organism is allowed to grow on this medium until one of the optical forms has been completely used. The optical form of the amino acid remaining in the solution is then isolated. Unfortunately, this method is of limited application since the organisms usually destroy the optical isomer occurring in nature and the unnatural one is left behind. In spite of this drawback, however, the method has been used quite often by Abderhalden and his co-workers in the study of the structure of amino acids.

The third method for separation of a racemic mixture of amino acids is the chemical method. This is by far the most applicable of the three methods although, in many cases, it is more tedious and difficult to use. The general procedure is to combine the amino acid or a derivative with some optically active compound. The alkaloids have been used very generally for this purpose since they are available in large quantities and in a relatively high degree of purity. Those usually employed are quinine, strychnine, brucine, and cinchonine. The alkaloids are basic. Many of the amino acids are not sufficiently acidic so that they can combine directly with the alkaloid, hence the benzoyl, acetyl, or formyl derivatives of the amino acids are used. In this way the basicity of the amino acid is masked and the resulting product is sufficiently acidic for combination. Usually beautifully crystalline compounds are formed. If

we select, for example, *l*-brucine and the racemic mixture of acetyl-dl-tryotophane, the resulting mixture of compounds formed will be composed of *l*-brucine-acetyl-d-tryptophane and of *l*-brucine-acetyl *l*-tryptophane. The relative solubility of these two compounds in most solvents is quite different and hence fractional crystallization of the mixture from a suitable solvent will enable one to make a sharp separation. The desired amino acid can be liberated first from the brucine and then from its acetyl derivative and thus obtained in a pure optically active form.

Besides the naturally occurring optically active alkaloids, some synthetic optically active organic compounds have been used for the resolution of amino acids. Acetyl-l-tryptophane has been racemized by the above procedure of racemization, using its sodium salt and an excess of acetic anhydride. The racemized mixture is then resolved with 4-methyl-phenyl-methyl-amine. The use of this compound could probably be applied to other racemic mixtures of amino acids.

Holmes and Adams (53) have attempted to use *l*-methoxy-acetyl chloride for the resolution of alanine, valine, phenylglycine, and phenylalanine. In the case of phenylalanine, the method was not successful due to the racemization of the two isomeric forms. Since the method has been applied only to the amino acids mentioned above and was not successful in one case, it is possible that the general applicability hoped for will not be attained.

d-Camphoric acid has been used by Berg (54) to resolve dllysine. The reagent combines with two molecular equivalents of the amino acid to form salts which can be fractionated by repeated crystallization from methanol-water mixtures. d-Lysine-d-camphorate is the more insoluble.

The resolution of many of the racemic amino acids is described by Fischer (55) and by Mahn (56).

SECTION II. THE PREPARATION OF AMINO ACIDS AND PROTEINS

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10. NATURALLY OCCURRING AMINO ACIDS

As has been pointed out in the earlier part of this chapter, it is necessary for many types of experiments, especially those of a biological nature, that the naturally occurring amino acids be used. In many instances the amino acid is more readily isolated from proteins than by resolution of the synthetic product. For the purpose of obtaining the best yields, it is always desirable to use protein material which is especially rich in the amino acid which is to be isolated. It is not the purpose here to outline in detail the procedures which must be followed in isolating individual amino acids, but rather to present the methods in a general way as well as the references to the literature so that those who may wish to prepare amino acids may do so more readily.

Glycine is best prepared synthetically (See Chapter II). Bergmann and Fox (126) isolated glycine from gelatin hydrolysates as a complex salt of potassium trioxalatochromiate. According to Towne (127) glycine forms a compound with nitranilic acid— $[(C_2H_5O_2N)_2\cdot C_6(OH)_2(NO_2)_2O_2)]$ —which may be used for its quantitative estimation.

The best source for *l-cystine* is human hair. Wool may also be used. The material is freed from fat by washing with gasoline. It is then hydrolyzed with strong hydrochloric acid until the biuret test is negative. The greater part of the acid is removed by vacuum distillation. Sodium acetate is added until the congo red test is negative. On standing at a low temperature, cystine, some of the tyrosine, and humin separate. The precipitate is filtered, dissolved in weak hydrochloric acid solution, the solution is decolorized by boiling with Norit, and the cystine is precipitated by addition of sodium acetate. The amino acid is washed free from inorganic salts with hot water and dried. The yield from hair is about 5 per cent (57). Instead of adding sodium acetate in the first step, excess of a suspension of lime may be used (58). This precipitates

the humin while cystine remains in solution. The greater part of the calcium hydroxide in the filtrate is neutralized with hydrochloric acid. Acetic acid is then added to bring the pH of the solution within the isoelectric zone of cystine (pH 3 to 6). The crude cystine is redissolved and reprecipitated as given above. A part of the *l*-cystine is destroyed during the hydrolysis and some may be racemized. Toennies and Bennett (59) prefer to hydrolyze the keratin with 50 per cent sulfuric acid. The greater part of the humin is removed by neutralizing the acid with sodium hydroxide to the turning point of congo red and saturating the solution with sodium sulfate. Excess of copper sulfate is added and the solution is again neutralized. The copper precipitate contains cystine, some leucine, aspartic acid, and humic acid. The copper salts are digested with hydrochloric acid and saturated with hydrogen chloride gas. l-Cystine hydrochloride crystallizes out on standing, while the inactive modifications remain in solution. Details of the method for preparing the isomers of cystine are given by du Vigneaud, Dorfmann and Loring (60), and by Toennies and Elliott (61).

d-Glutamic acid is best isolated from the gluten of wheat flour (62). Since the monosodium salt is produced commercially in large quantities (63) and may be purchased quite cheaply (Ajinomoto, Kin-Saji, Maywesuit), it is more convenient to prepare the amino acid from this source (64). The free acid is isolated by adding sufficient hydrochloric acid to bring a concentrated solution of the monosodium salt to pH 3.2, the isoelectric point of glutamic acid. The free amino acid crystallizes out at low temperatures. Care must be taken not to convert the glutamic acid to l-pyrrolidone carboxylic acid by heating (65).

l-Tyrosine may be isolated from either silk waste (66) or casein (67). If silk is used, hydrolysis is effected by means of hydrochloric acid. As much of the acid as possible is then removed from the filtered solution by vacuum distillation, the solution is decolorized, and the pH is brought to 5.7, the isoelectric point of this amino acid. Crystals are obtained on permitting the solution to stand in the cold. Purification is effected by recrystallization from water. The yield is 8 to 10 per cent. If casein is used, the protein is dissolved with the aid of ammonia. The mixture is digested with trypsin. A part of the tyrosine will crystallize out without adjusting the pH.

¹ North American Mercantile Company, 330 Front Street, San Francisco, California.

The rest is obtained on addition of acetic acid to the isoelectric point of this amino acid. The remainder of the procedure is the same as that which is given above. The yield of tyrosine is about 4 per cent. When prepared in the manner given above, tyrosine is invariably contaminated with leucine. The latter amino acid may be removed by refluxing the mixture of the two amino acids with glacial acetic acid which dissolves the leucine (68). l-Diiodotyrosine may be prepared from l-tyrosine according to the procedure of Oswald (69), l-dichlorotyrosine and l-dibromotyrosine according to the procedure of Zeynek (70).

l-Tryptophane is best prepared from a tryptic digest of casein. The filtrate, after removal of the greater part of the tyrosine and some of the leucine, may be used for this purpose. The tryptophane is precipitated by the addition of a sulfuric acid solution of mercuric sulfate. Small amounts of tyrosine are removed from the precipitate by washing with a mercuric sulfate solution. The precipitate is suspended in water, made alkaline to phenolphthalein with barium hydroxide, and decomposed with hydrogen sulfide. The barium is precipitated with sulfuric acid, the filtrate is concentrated, and the amino acid is extracted with butyl alcohol. The produce is recrystallized from dilute ethyl alcohol. The yield is about 0.6 per cent (71) (66 b).

The isolation of *l-methionine* has been described by Pirie (72) and by du Vigneaud and Meyer (73). The procedure used by Pirie includes extraction of a casein hydrolysate with butyl alcohol, precipitation of methionine by means of an acetic acid solution of mercuric acetate, and, after decomposition of the mercury compound with hydrogen sulfide, the hydrochloric acid in the solution is neutralized with pyridine. On cooling the solution, methionine crystallizes out. The product obtained contained 95 per cent of methionine and the yield was 1.4 per cent. In the second mentioned procedure casein is digested with pancreatin. After removing tyrosine and tryptophane, additional mercuric sulfate and sulfuric acid are added and the reaction is adjusted to the turning point of congo red by addition of sodium hydroxide solution. The precipitate is filtered, suspended in water, and decomposed by adding barium hydroxide in excess. After removal of barium ions, the methionine is precipitated by addition of mercuric chloride. After decomposing the precipitate with hydrogen sulfide, aniline is added to the concentrated solution in order to neutralize the hydrochloric acid. The methionine is recrystallized from its aqueous solution by addition of alcohol. The yield of analytically pure methionine was about 0.1 per cent. None of these procedures affords a ready method for preparing methionine in large quantities. This is due, in part, to the low content of methionine in casein. The purity of methionine preparations should always be ascertained. The product is often contaminated with phenylalanine and leucine.

The isolation of *l-proline* from protein hydrolysates, according to the method described by Towne (74), depends upon the fact that the copper salts of proline, hydroxyproline, valine, and possibly several other amino acids are soluble in both water and methyl alcohol. On the other hand, certain of the amino acids yield copper salts which are soluble in water but insoluble in methyl alcohol, while the copper salts of the rest are insoluble in both water and methyl alcohol. After decomposing the copper salts, which contain proline, with hydrogen sulfide and concentrating, proline is extracted with absolute alcohol. The alcohol is distilled off and proline is precipitated from the aqueous solution as the picrate. Cox and King (75) recommend the use of aniline for decomposing proline picrate. If used in excess it will dissolve the aniline picrate and thus afford a way of removing this compound from the aqueous solution of the amino acid. Proline may be extracted directly with absolute alcohol from a gelatin hydrolysate and subsequently converted into the picrate. Either wheat gliadin or gelatin may be used as a source for proline. The yield is nearly quantitative. Bergmann (128) uses ammonium rhodanilate to isolate proline. Arginine is removed from a hydrochloric acid hydrolysate of gelatin and ammonium rhodanilate is added as long as a precipitate is formed. The proline rhodanilate is decomposed by addition of pyridine. From 100 gms. of gelatin 97 gms. of l-proline rhodanilate were obtained. Hydroxyproline may be isolated as the reineckate after removal of arginine and proline. The yield of hydroxyproline is about 10 per cent.

Since *l-hydroxyproline* also yields a copper salt which is soluble in both water and methyl alcohol, its isolation can be undertaken along with that of proline (76). The two amino acids can be separated from each other by means of absolute alcohol. Hydroxyproline is isolated as the picrate from the alcohol-insoluble residue. The yield of hydroxyproline, when isolated from gelatin, is about 2 per cent. Both proline and hydroxyproline yield no nitrogen when treated with nitrous acid.

A number of procedures for the isolation of the basic amino

acids have been described. Hanke and Koessler (77) (66 b) use mercuric chloride for the isolation of *l-histidine* from a hydrolysate of blood corpuscle paste. The yield from 500 cc. of paste is about 25 gms. of histidine dihydrochloride. Leucine and tyrosine may be isolated as by-products. *d*-Arginine monohydrochloride may be isolated from a gelatin hydrolysate by means of flavianic acid. Arginine flavianate is decomposed by addition of hydrochloric acid, and the excess of acid beyond that which is required for the formation of arginine monohydrochloride is neutralized with aniline. The details are given by Cox (78). The yield of arginine monohydrochloride is 7 to 8 per cent. The basic amino acids may also be prepared by the quantitative procedure which is described in Chapter IV.

Perhaps one of the best methods of preparing l-histidine, d-arginine, and d-lysine is by the method of electrical transport which has been discussed in the first section of this chapter. l-Leucine¹ and l-tyrosine may be isolated incidentally. Cox, King, and Berg (37) have described the details of the technique. At pH 5.5 the three hexone bases migrate to the cathode compartment of the three-compartment cell when a direct current is passed through the solution. Arginine and lysine may be separated from histidine by subjecting a solution of the three amino acids to electrical transport at pH 7.5 (36). Arginine is precipitated as the flavianate. The filtrate is freed of flavianic acid by the addition of barium hydroxide, and the excess of barium ions is removed with sulfuric acid. The solution is concentrated, alcohol is added, and lysine picrate crystals are obtained on addition of a solution of picric acid in alcohol. Lysine picrate is decomposed by adding hydrochloric acid. The picric acid is extracted with benzene. Histidine is precipitated from its solution by addition of mercuric sulfate and subsequently isolated in the usual manner as the dihydrochloride. By adjusting the pH to the isoelectric points, leucine and tyrosine may be crystallized out in large part from the protein hydrolysate before subjecting it to electrical transport. The separation of the two amino acids is made by extracting the solids with glacial acetic acid. Cox, King, and Berg report a yield of 51 gm. of histidine monohydro-

¹ *l-Leucine* is usually contaminated with methionine. According to Fox (Fox, S. W., *Science*, **84**, 163 (1936)), leucine may be freed of methionine by formylating the mixture of the two amino acids. Two recrystallizations from water render the formyl-leucine practically sulfur-free. The formyl-leucine is then decomposed with hydrobromic acid and the free amino acid is crystallized out of the solution. The loss of leucine may be as much as 50 per cent.

chloride, 21 gm. of arginine monohydrochloride, 62 gm. of lysine dihydrochloride, 58 gm. of leucine, and 22 gm. of tyrosine from 4 kilos of blood corpuscle paste. The isolation of free arginine, histidine, and lysine has been described by Vickery and Leavenworth (79). It should be mentioned that in preparing leucine, there is always a possibility that the product obtained is contaminated with methionine or one of the leucine isomers.

l-Aspartic acid may be obtained along with glutamic acid when these amino acids are isolated as the alcohol-insoluble barium salts. A more convenient way of preparing aspartic acid is by hydrolysis of asparagine with dilute hydrochloric (80) or nitric acid (81). The mineral acid is subsequently neutralized by addition of ammonia to pH 2.8, the isoelectric point of the amino acid, and the aspartic acid is permitted to crystallize. The amino acid may be freed of ammonium salts by extracting it with methyl alcohol and recrystallizing. The yield is 80 to 90 per cent.

d-Alanine can be isolated from silk fibroin. After hydrolysis of the protein, the excess of sulfuric acid is neutralized in the usual manner and tyrosine is crystallized out. The remainder of the amino acids is esterified, glycine ester hydrochloride is removed by crystallization, and the ester of alanine is obtained by fractional distillation. This method possesses the disadvantages which are inherent in the process of esterification and the separation of the amino acid esters.

Thyroxine can be isolated from the thyroid gland by hydrolyzing the protein with barium hydroxide. The details of the method have been described by Harington (83). The racemic form of the amino acid is obtained. Harington (84) has described a method for resolving it into its optically active components. Small amounts of *l*-thyroxine have been obtained by hydrolyzing the thyroid protein with pepsin and trypsin (85).

The isolation of serine from sericin has been described by Daft and Coghill (82). The protein is hydrolyzed with 25 per cent sulfuric acid. Sufficient barium hydroxide is added to bring the reaction to pH 9.0. After the mixture has stood for one hour the reaction is adjusted to neutrality with sulfuric acid, and the filtrate, after removal of barium sulfate, is concentrated. Crystals of tyrosine, alanine, and serine appear successively. Serine is recrystallized by adding alcohol to the hot aqueous solution of the amino acid.

Serine is racemized by the addition of barium hydroxide to pH 9.0, and it is therefore necessary, in order to obtain *l*-serine, to

resolve the synthetic product. Attempts to isolate the optically active amino acid from the protein hydrolysate have not been particularly successful on account of the solubility of *l*-serine. Racemic serine is much less soluble than the optically active form. Since *l*-serine is not isolated directly, the method leaves much to be desired.

The preparation of the other amino acids in considerable amounts from natural sources is in a less satisfactory state. When the naturally occurring form of the amino acid is desired, recourse is usually had to resolution of the synthetic product. References giving the sources from which this group of amino acids has been isolated are quoted by Mahn (56a) and by Vickery and Schmidt (86).

11. PROTEINS

Many of the proteins, both of animal and plant origin, when prepared by different workers, have the same properties and thus appear to be pure compounds. The name of a particular protein, however, does not indicate that, from a chemical standpoint, we are dealing with a single entity. In fact, quite the converse may, at times, be true. For example, it has been shown that casein is a mixture of several proteins. The protamins are likewise not homogeneous chemical compounds, nor is gelatin. Numerous other examples could be cited. On the other hand, as far as our present knowledge extends, some of the animal proteins, such as egg albumin, hemoglobin, and also some of the vegetable proteins, consist of a single type of molecule.

Despite the uncertainties which may exist concerning the homomolecularity of many of the proteins, they can still be used for certain types of experiments. Many of the data which relate to the behavior of proteins in general have been obtained by the use of non-homogeneous proteins. Certain of these proteins possess characteristics, such as an excess of acidic or basic groups, ability to form gels, etc., which make them exceedingly useful for particular experimental purposes.

For physical chemical experiments, it is usually necessary that the protein be salt-free or at least that the content of ash be reduced to a minimum. In the case of proteins which are insoluble at their isoelectric points, it is possible to reduce the ash content to a low level. This may be accomplished, in large part, by washing the isoelectric protein repeatedly with distilled water. Further removal of salts may be effected by electrodialysis. This is carried

out by placing the protein in the center compartment of a threecompartment cell, using parchment paper or cellophane to separate the compartments. The cathode and anode compartments contain running distilled water. Electrodes of sheet carbon may be used. On passing a direct current through the cell, the concentration of inorganic electrolytes may be brought to a low level. In the case of proteins which are extracted from natural products by means of a solution of sodium chloride or, in the case of proteins which are precipitated by half or complete saturation with ammonium sulfate, the greater part of the inorganic electrolyte may be removed by placing the protein in a parchment or cellophane bag and dialyzing first against running tap water, and later against distilled water. Bacterial growth is inhibited by carrying out the dialysis at low temperatures, or by the use of toluol. The dialysis may be expedited by stirring the contents of the bag mechanically. At times it may be preferable to dialyze the protein solution against a buffer solution whose pH is the same as the isoelectric point of the protein, before dialyzing against distilled water.

It is often desirable to prepare the protein in a dry state. In certain instances this may lead to denaturation. However, if this is not a factor which must be considered, dehydration may be carried out in the following manner. The salt-free precipitate of protein is washed repeatedly, first with 95 per cent alcohol, and finally with absolute alcohol. The supernatant fluid is removed each time by decantation. Absence of water in the supernatant fluid is determined by testing a portion with anhydrous copper sulfate. The major portion of the alcohol is removed by washing the protein repeatedly with anhydrous ether. The product is quickly filtered on hardened filter paper and dried over sulfuric acid. If care is taken to remove all of the water, the product obtained consists of a light powder resembling flour. If, however, appreciable amounts of water are present, the product will be converted, during the drying procedure, to hard lumps which are difficult to grind. In the case of proteins which crystallize easily, purification may be effected by recrystallization. Insoluble material may be removed by filtration with suction through washed filter paper pulp.

Many of the principles which underlie the preparation of the proteins listed below are given in Chapter VII.

Most of the vegetable proteins are obtained from the seeds of plants. The principles which underlie the methods for isolating the vegetable proteins are given by Osborne (87). We may illustrate

certain of the techniques used in preparing vegetable proteins by using edestin and gliadin as examples. While it is possible to extract edestin from hemp seed without previously removing fat and chlorophyll, it is, however, best to remove them. This may conveniently be done by extracting the ground seed with ether, using the extraction apparatus which is shown in Fig. 14. The fat-free

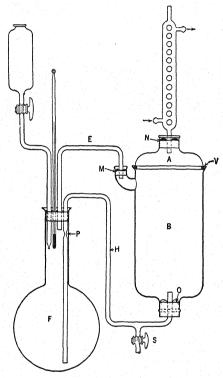


Fig. 14. Fat extractor. (Schmidt, C. L. A., Ind. Eng. Chem., 8, 165 (1916).)

meal is macerated with 10 volumes of 5 per cent sodium chloride solution at about 50° for an hour and filtered at this temperature through soft filter paper. The reaction of the extract is adjusted to pH 6.9, the isoelectric point of edestin. The solution may be dialyzed or permitted to stand in the refrigerator overnight. Crystals of edestin separate. The supernatant fluid is syphoned off, the precipitate is redissolved in warm sodium chloride solution, filtered, and recrystallized. This process is repeated several times. The crystals are washed repeatedly with carbon dioxide-free distilled water at a low temperature. Electrodialysis may be used to

reduce the content of ash. The product is finally dehydrated by washing with alcohol and ether and dried over sulfuric acid. The electrically heated vacuum desiccator described by Robertson and Schmidt (88) may be used for this purpose. According to Osborne the composition of edestin is: C=51.3, H=6.8, N=18.8, S=0.9, and O=22.2.

The preparation of gliadin has been described by Dill and Alsberg (89). Wheat flour is moistened with water so as to form a dough. This is washed free from starch by kneading it in running water. The moist gluten is extracted repeatedly with 70 per cent (by volume) alcohol. The filtered extract is concentrated in vacuo below 50°. The concentrated syrup is poured into 5 volumes of one per cent aqueous sodium chloride solution. On shaking, the gliadin is precipitated as a foam. This is dissolved in 70 per cent alcohol. On standing in the refrigerator much of the gliadin separates. The precipitate carries down most of the lipids and suspensoid impurities. It is dissolved and reprecipitated as before. The crude gliadin is finally purified by pouring the concentrated alcohol-containing gliadin syrup into a one per cent aqueous solution of lithium chloride and washing the precipitate repeatedly with this solution. The wet gliadin is dissolved in the minimum quantity of alcohol. It is reprecipitated by pouring the solution into four volumes of absolute alcohol, containing 0.025 per cent lithium chloride. The process of dissolving the gliadin in alcohol and precipitating it from a stronger concentration of alcohol is repeated several times. The final precipitate is freed of water by grinding and washing it repeatedly with absolute alcohol and then with ether. It is dried in vacuo. When prepared in this manner, gliadin forms water-clear solutions when dissolved in 50, 60, and 70 per cent alcohol. The nitrogen content of gliadin is about 17.54 per cent, and the ash is about 0.08 per cent. On acid hydrolysis 26.2 per cent of ammonia nitrogen is obtained.

The essential features of the above method are: (a) precipitation of the gliadin in aqueous solution thus permitting thorough washing of the foamy precipitate, and (b) use of lithium chloride instead of sodium chloride to promote precipitation. Since lithium chloride is soluble in both alcohol and ether, it is possible to obtain preparations of gliadin which are practically ash-free. Lithium chloride might well be used to promote precipitation as well as solution of other proteins. By its use one may, at times, dispense with dialysis.

Casein is one of the most useful proteins for certain types of investigative work. One of the more recent descriptions for preparing it is given by Cohn (90). The casein is precipitated from skimmed milk by addition of 0.05 N HCl, with rapid stirring, to pH 4.6, and the precipitate is washed repeatedly by decanting with distilled water. It is then slowly dissolved by addition of 0.1 N sodium hydroxide to pH 6.3. The solution is filtered and the casein is again precipitated by addition of hydrochloric acid with rapid stirring as before. The precipitate is washed free from chlorides with distilled water and the water is removed by washing with alcohol and ether.

When prepared according to the above procedure, casein consists of at least three, and possibly four, proteins which can be separated from each other by fractionation with suitable solvents (91), (92), (93), (94). The preparation of other proteins which are present in milk is described by Pedersen (95).

The preparation of low-ash *gelatin* is a fairly simple procedure. Granulated gelatin is repeatedly washed with M/128 acetic acid at a low temperature. This is followed by washing with distilled water and alcohol. Another procedure is to wash the granules a number of times, first with a 10 per cent sodium chloride solution containing 5 cc. of concentrated hydrochloric acid per liter, then with weaker concentrations of sodium chloride solution without acid, and finally with distilled water until the wash water is free from electrolytes. This is followed by treatment with alcohol and ether in the usual manner. The use of dilute lithium chloride solution, before washing with distilled water is begun, may facilitate the subsequent removal of the electrolyte. The basis of this procedure is removal of ash from a water-insoluble protein by washing it at the isoelectric point. Since the isoelectric point of gelatin, pH 4.7, lies in the acid region, there is little difficulty in removing such contaminants as calcium phosphate (96), (97), (98). An analysis of gelatin by Smith (98) gave the following percentages: C = 50.5, H = 6.8, N = 17.5, O = 25.2.

Gelatin consists of a number of different proteins with molecular weights ranging from 11,000 to 70,000. It has not been possible to separate a definite chemical species from gelatin (99).

The various methods which have been described for the preparation of egg albumin are essentially minor modifications of the technique which was used by Hopkins and Pinkus (100) and Hopkins (101). Details of the technique are given by Sörensen and Höyrup (102), Taylor, Adair, and Adair (103), and La Rosa (104). The globulin fraction is precipitated by addition of ammonium sulfate to one half saturation. The egg albumin is crystallized by saturating the solution with ammonium sulfate and adjusting the pH to 4.8, the isoelectric point of this protein, by the addition of dilute sulfuric or acetic acid solution. The electrolyte is removed by dialysis. At least three recrystallizations are necessary in order to remove all of the ovomucoid and conalbumin. The crystals may be washed several times with saturated ammonium sulfate solution containing acetic acid-sodium acetate buffer without appreciable loss of protein. Dialysis may be carried out first against a sodium and potassium phosphate buffer solution, then against distilled water, and finally the last traces of electrolyte may be removed by electro-dialysis. The solution of egg albumin may be kept unaltered by the addition of toluol or by refrigeration. Flosdorf and Mudd (105) report that this protein, as well as others, may be preserved for long periods of time by their method of rapid freezing and dehydration.

Egg albumin has a nitrogen content of 15.6 per cent. It is one of the proteins which is considered to be homomolecular.

Blood serum yields a number of proteins. Serum globulin may be prepared by adding an equal volume of saturated ammonium sulfate solution to blood serum, centrifuging off the precipitate, dissolving the precipitate in water, and reprecipitating. The last procedure is repeated three or four times. Dialysis is carried out against a phosphate buffer solution of pH 5.5 at 0°. The protein may be kept under toluol in a phosphate buffer solution (0.19 M KH₂PO₄ and 0.009 M Na₂HPO₄) at a low temperature. The nitrogen content of horse serum globulin is 15.1 per cent. According to Svedberg and Sjögren (106), serum globulin is a homogeneous protein. It is unstable, easily breaking up into euglobulin and pseudoglobulin. McBain and Jameson (107) believe that globulin, euglobulin, and pseudoglobulin represent three phases of a system of the same parent substance, dehydrated globulin. A solution of globulin represents the ordinary isotropic phase, while euglobulin and pseudoglobulin are liquid crystal phases.

Serum albumin is prepared in much the same manner as egg albumin. After removal of the globulin fraction crystallization of the serum albumin may be brought about by the addition of dilute acetic or sulfuric acid dissolved in a solution of ammonium sulfate. The crystals are washed with a dilute solution of ammonium sulfate.

They are then dissolved in distilled water and reprecipitated. The recrystallization should be repeated several times. Dialysis is carried out first against a phosphate buffer solution, then against distilled water, and finally electrodialysis is employed to remove the last traces of electrolyte. The nitrogen content of horse serum albumin is 15.6 per cent. Its isoelectric point is about pH 5.5. According to Svedberg and Sjögren (106), serum albumin is a homogeneous protein. Details of the method of preparing serum albumin and serum globulin are given by Svedberg and Sjögren (106) and by Adair and Robinson (108).

In preparing hemoglobin advantage is taken of the fact that certain hemoglobins, viz., those of the horse and dog, are insoluble at the isoelectric point and hence may be easily crystallized. Hemoglobin from the blood of man, the ox, and the sheep is quite soluble in water in its isoelectric condition. It is usually best to wash the red cells repeatedly with isotonic salt solution to free them of serum proteins. The cells are laked by addition of distilled water or distilled water and ether. The stromata are removed by centrifuging. Crystallization may be effected by lowering the temperature to 0° and treating the solution with a mixture of carbon dioxide and oxygen (109). In methods of this type some of the hemoglobin is altered so that its oxygen-binding capacity is diminished.

In the method of Stadie and Ross (110) the washed red cells, or a solution of hemoglobin freed from stromata, are subjected directly to electrodialysis. After several hours hemoglobin crystals are formed. Recrystallization with a loss of about 30 per cent may be carried out. The amount of base bound to the hemoglobin may be appreciably reduced by passing a stream of carbon dioxide through the solution during electrodialysis. The hemoglobin is best preserved in the moist state at a low temperature. Even then there is a slow conversion into inactive hemoglobin. Drying leads to a similar change. The concentration of hemoglobin in an aqueous solution may be determined by the method of Stadie (111). Other methods of preparing hemoglobin are given by Adair and Adair (112). Marshall and Welker (113) remove proteins other than hemoglobin from laked blood by the use of aluminum hydroxide. Red blood cells also contain a carbon dioxide-precipitable globulin. Methods for its isolation are given by Bennett and Schmidt (114).

Methods for preparing globin have been described by Robertson (115), Hill and Holden (116), Holden and Freeman (117), Anson and Mirsky (118), and others (119). The procedure used by Anson

and Mirsky consists in adding hydrochloric acid and acetone-containing hydrochloric acid to an aqueous solution of carbon monoxide hemoglobin. A precipitate of globin hydrochloride and a solution of heme are obtained. After removal of acetone, the globin powder readily dissolves in water and yields an acidic reaction. Globin may be completely precipitated from this solution by addition of ammonium sulfate to one-third saturation or almost completely precipitated by rapid and complete neutralization. The precipitate has the characteristics of denatured protein. It is insoluble in water at pH 8.0, the isoelectric point of globin. It combines with reduced heme to form hemochromogen.

If the solution of globin hydrochloride is gradually neutralized, the greater part of the globin is converted into a form which is soluble in a neutral solution 0.4 saturated with ammonium sulfate. This form of globin possesses the properties of native globin. It is soluble in distilled water, it can be coagulated by heat, and it can combine with heme to give a product which has the spectrum of methemoglobin. On reduction, the reduced form of hemoglobin is obtained. When a solution of this product is shaken with air it yields the spectrum of oxyhemoglobin.

Fibrin (120), (121) is prepared by permitting blood to clot and removing the clot, or by whipping the blood and removing the shreds. This material is washed repeatedly in water to remove the soluble proteins. Sodium hydroxide solution is now added and the mixture is warmed to about 60°. After filtration the fibrin is precipitated by addition of acetic or hydrochloric acid until the maximum flocculation occurs. The process of solution and precipitation is repeated several times. The fibrin is finally washed free from electrolytes with distilled water and dehydrated with alcohol and ether. According to Bosworth (120), the nitrogen content is 17.2 per cent. The isoelectric region is pH 4.5 to 6.7. Greenberg (121) reports that the electrochemical equivalent of fibrin varies with the mode of preparation.

Fibrinogen may be isolated from either oxalated plasma or from blood to which 25 per cent magnesium sulfate solution (one volume magnesium sulfate solution and four volumes of blood) has been added. The blood is centrifuged and the clear plasma is removed. The fibrinogen is salted out by adding an equal volume of a saturated solution of sodium chloride. The precipitate is washed with distilled water. It is then dissolved in M/6 saline solution and reprecipitated by addition of sufficient solid sodium chloride to make

a 15 per cent solution. The electrolyte is finally removed by dialysis. The isoelectric point of fibrinogen is 5.5. The details of the procedure are given by Nordbö (122).

The protamins are present in ripe fish sperm combined with nucleic acid. Clupein is obtained from the sperm of herrings, salmon sperm yields salmin, and sturgeon sperm, sturin. The method of preparation is essentially that described by Kossel (123). The ripe testicles are shaken with water, the suspension of sperm is strained, and the fluid is coagulated by addition of acetic acid. The precipitate is dehydrated with alcohol and ether. The dried material is extracted repeatedly with a one per cent solution of sulfuric acid and the protamin sulfate is precipitated by addition of alcohol. Further purification is effected by dissolving the protein in water, filtering, and precipitating it by addition of alcohol. The last traces of nucleic acid may be removed by precipitating the protamin with sodium picrate. The picric acid is subsequently removed by addition of sulfuric acid and extraction with ether. The protamin sulfate is precipitated by addition of alcohol.

According to Felix and his co-workers (124) clupein is not a chemical entity but consists of at least four protamins which have been termed A_1 , A_2 , B, and C.

It is doubtful that any of the *keratins* which have been isolated represent homogeneous molecular species. However, for certain types of experimental work, partially purified products are found useful. Goddard and Michaelis (125) prepare wool keratin by dissolving wool in a solution of sodium thioglycolate (pH 12). The solution is filtered and the keratin is precipitated by adding acetic acid. The precipitate is filtered off, ground with acetone which is acidified with hydrochloric acid, washed with ether, and dried. The last traces of thioglycolic acid may be removed by dialysis. The keratin is insoluble in water but can be dissolved by the addition of sodium bicarbonate or sodium carbonate solution. The ability of alkaline sodium sulfide, potassium cyanide, or thioglycolic acid solutions to dissolve keratin appears to depend on the splitting of the disulfide groups which are essential for the maintenance of the fibrous structure of keratin.

Lack of space prevents enumeration of the references in which the preparation of other proteins is given. The preparation of some of the proteins not listed here is given by Svedberg and his coworkers (see Chapter VII, Table XIV). Reference to others may be obtained from Chemical Abstracts.

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CHAPTER IV

METHODS OF ANALYSIS AND REACTIONS OF THE AMINO ACIDS AND PROTEINS

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The properties of proteins depend on the presence of characteristic groupings in the amino acids and on the nature of the linkages between them. The methods of analysis which are applicable to proteins are based largely on the reactions of the specific groups in the constituent amino acids and on the nature of the linkages between them. The reactions which are most extensively used involve color changes in the presence of certain reagents. These tests are frequently quite specific for particular amino acids. The peptide linkage, which is the linkage between the amino group of one amino acid and the carboxyl group of another, may also be recognized by a characteristic color reaction.

1. COLOR REACTIONS

(1) Millon's Reaction.¹ When a protein solution is treated with a few drops of Millon's reagent, a white precipitate is usually formed which, on warming, becomes brick red. Millon's reagent contains mercurous and mercuric nitrate in a solution of nitric acid. The reaction which occurs between the protein and this reagent is probably due to the formation of a nitro derivative of the protein (if a hydroxyphenyl group, as in tyrosine, is present), and the subsequent formation of a mercury salt. The only amino acid which has been obtained from a protein hydrolysate which gives this color reaction is tyrosine. It is concluded from this, therefore, that any protein which gives the Millon test contains tyrosine, and that one which does not give the test does not contain tyrosine. The reaction, however, is not specific for tyrosine. For example, phenol will give the test and so will salicyl aldehyde, phloroglucinol, catechol,

¹ See Cole, S. W., Practical Physiological Chemistry, 9th ed., Cambridge 1932, for recent modifications in the technique of carrying out the Millon and the Hopkins-Cole tests.

and other hydroxyphenyl derivatives which have been tested. It is concluded that this reaction is specific only for the hydroxyphenyl group. The reaction is very sensitive when properly carried out. It has been used not only for the qualitative detection of tyrosine in the protein molecule, but also as a basis for a very excellent quantitative colorimetric method. The test, however, is not entirely satisfactory for use on solutions which contain large quantities of inorganic salts, particularly chlorides, since the mercury is usually precipitated and the reagent is rendered inert. The method is, therefore, not applicable to the determination of tyrosine in the urine. It is also not applicable in the case of solutions which contain large quantities of alkali.

- (2) The Xanthoproteic Reaction. When concentrated nitric acid is added to most protein solutions, a white precipitate forms which, on heating, turns yellow and usually redissolves. When a strong alkali such as sodium hydroxide is added to such a solution, the vellow color deepens to an orange color. It is believed that this reaction is due to the presence of the phenyl group in the protein molecule with which the nitric acid reacts to form nitro compounds. The amino acids which contain this grouping and which occur generally in proteins are tyrosine and tryptophane. Diiodotyrosine and thyroxine may, at times, also be present in proteins and will respond to this test. Most proteins give the xanthoproteic reaction. Among those which do not respond are some of the protamins which do not contain tyrosine and tryptophane. Gelatin gives only a faint color. This reaction likewise cannot be used for the detection of phenolic compounds in urine because the color of the urine interferes with the color of the end product.
- (3) The Hopkins-Cole or Glyoxylic Acid Reaction. The reagent contains glyoxylic acid. When a solution of tryptophane or a solution of a protein which contains tryptophane is mixed with a solution of this reagent, and the mixture is placed above a layer of concentrated sulfuric acid, a violet ring appears at the interface of the two liquids. Among the amino acids this color reaction is specific for tryptophane. Other compounds which contain the indole ring will also react. Gelatin and some of the protamins which do not contain tryptophane fail to respond to the test. This test can be used for solid proteins as well as for protein solutions, provided that, in the former instance, the solid is placed at the zone of contact between the reagent and the concentrated sulfuric acid.

(4) The Ninhydrin Reaction.^{2,3} When amino acids which contain a free amino group in the α -position are allowed to react with

blue color is formed. β - and γ -amino acids, and α -amino acids in which the amino group is substituted, do not react. A free amino group and a free carboxyl group must be present in the molecule in

² D. D. Van Slyke and R. T. Dillon (*Compt. rend. Lab. Carlsberg*, **22**, 480 (1938)) use ninhydrin for the quantitative determination of carboxyl groups in amino acids. The carbon dioxide of α -amino acids is liberated in 3 minutes. Aspartic acid, however, yields 1.9 moles and glutamic acid 1.03 moles of carbon dioxide.

³ The reaction between ninhydrin and α -amino acids involves two steps. The first step involves dehydrogenation of the amino acid with the formation of the

aldehyde, ammonia, and carbon dioxide:

$$C(OH)_2 + R \cdot CH(NH_2) \cdot COOH \rightarrow COOH \rightarrow COOH + R \cdot CHO + NH_3 + CO_2$$

$$C \cap COOH \rightarrow COO$$

Ninhydrin and its reduction product then condense with ammonia to yield the blue colored compound:

The constitution of this compound is analogous to that of murexide,

$$O=C$$
 $NH \cdot CO$
 $C-N=C$
 $CONH$
 $C=O$
 $NH \cdot C(OH)$

(See Retinger, J., J. Amer. Chem. Soc., 39, 1059 (1917).)

order to obtain the color. For qualitative purposes the ninhydrin test is one of the most delicate reactions known for the determination of the presence of proteins and α -amino acids in fluids. By means of this reagent, the presence of amino acids in fresh urine and in de-proteinized serum can be shown. So far as is known, all of the amino acids, except proline and hydroxyproline, which have been obtained from protein hydrolysates give this reaction. The reaction can be used for the quantitative determination of an amino acid when that amino acid alone is present in the solution. It is not, however, applicable to the quantitative determination of amino acids in a mixture due to the fact that various amino acids give differences not only in shade of color, but also in depth of color. Thus, if alanine is used as a standard, small amounts of alanine can be determined accurately. If, however, the attempt is made to determine glycine or phenylalanine by means of an alanine standard, the values will be incorrect. This method is often used to determine the end-point of a protein hydrolysis, since the color value at the end of the hydrolysis is constant.

(5) The Biuret Reaction. This reaction is given by, but not specific for, the peptide linkage. Peptides and proteins give the reaction. However, some compounds, for example, histidine, which does not contain the peptide linkage, also respond to the test. The reaction is given by all substances which contain two amino groups in their molecules, these groups being joined either directly or through a single atom of nitrogen or carbon. The reaction derives its name from the fact that, when urea is heated at about 180°,

biuret,
$$\| \| \|_{NH_2-C-NH-C-NH_2}$$
, is formed and responds to the test.

The test is carried out by mixing thoroughly the substance to be tested with strong potassium or sodium hydroxide and slowly adding a very dilute solution of copper sulfate until a purplish or pinkish violet color is obtained. The nature and the depth of the color depend upon the type of compound which is being investigated. Proteins give a purplish violet color, proteoses and peptones give a decided pink color, simple peptides give a very light pink color, while the color which is produced with gelatin is almost blue. The biuret test probably depends upon the formation of a definite copper compound (12):

If magnesium sulfate is present in the solution under test, magnesium hydroxide is formed and interferes with the reaction. If large amounts of ammonium sulfate are present, a large excess of alkali must be used. A single peptide linkage, such as that which is present in glycyl-glycine, does not give the biuret reaction. Three or more amino acids must be linked in order to obtain a positive biuret test. The test is of value in determining the presence or absence of small amounts of protein in biological fluids. The fact that this reaction is characteristic of all proteins and of all compounds which contain three or more amino acids linked through the carboxyl and the α -amino groups is strong support for the theory that the peptide linkage is the primary, if not the only, linkage in the protein molecule which exists between the amino acids.

- (6) Folin's Reaction. A general colorimetric reagent for the estimation of the amino acids was described by Folin (13). It contains 1,2-naphthoquinone-4-sulfonic acid. In the presence of alkali this reagent gives a striking, deep red color with amino acids. Urea, uric acid, creatinine, creatine, and hippuric acid do not respond to the test, while ammonia does so. Fortunately, when present in solutions of amino acids, ammonia can readily be removed. A number of nitrogenous bases, such as the alkaloids and amino-benzene derivatives, give positive reactions. These are generally not encountered when the presence or absence of amino acids in fluids is to be determined.
- (7) The Benzaldehyde Reaction. A specific color reagent for the indole nucleus and, therefore, for tryptophane is a solution of p-dimethylamino-benzaldehyde (Ehrlich's reagent). When a protein or a mixture of amino acids containing tryptophane, or any

compound which contains the indole nucleus, is treated with Ehrlich's reagent in the presence of strong hydrochloric acid, an intense dark blue color develops. This color is usually quite stable and lends itself readily to the quantitative determination of compounds which contain the indole nucleus. This reaction is merely another modification of the tryptophane aldehyde reaction which has already been illustrated by the Hopkins-Cole reagent. The test has often been used to determine bacterial infection, since many of the microorganisms have the ability to form indole from compounds which do not contain the indole nucleus.

(8) The Sakaguchi Reaction. Sakaguchi, in 1925 (14), observed an extremely sensitive color when certain guanidine-containing compounds reacted with α -naphthol and sodium hypochlorite. This reaction has been extensively employed as a qualitative test for arginine. The color is an intense red which develops very slowly, with probable destruction of part of the guanidine group. The reaction is probably specific for arginine since it is the only amino acid containing the free guanidine group which so far has been found in protein hydrolysates. A modification of the original Sakaguchi reaction in which sodium hypochlorite has been replaced by sodium hypobromite has been described (15). When sodium hypobromite is used, the color development with arginine is practically instantaneous. However, quantitative colorimetric estimations with this reagent are not possible because the color fades and disappears almost entirely within ten minutes. For purposes of stabilizing the color in order to permit colorimetric comparisons, urea is added immediately after the color develops.

Although arginine is the only naturally occurring amino acid which responds to this reaction, other guanidine derivatives which likewise give this test, occur in nature. The following compounds are known to give an intense red color in the modified method: arginine, glycocyamine, methylguanidine, and argininic acid; while creatine, creatinine, urea, glycocyamidine, guanidine, and assymetric dimethylguanidine do not yield a color. It is predicted

that derivatives which have the formula, XHN-C-NH₂, where X

is either a fatty acid or alkyl radical) will give a color with this reagent.

Arginine reacts positively with the Sakaguchi reagent in a dilution of 1:2,500,000 or 0.0004 mg. per cc. Although proteins give

the reaction because of their arginine content, the colorimetric determination of arginine in unhydrolyzed proteins is not possible since the arginine does not give its maximum color when in combination. Since the color intensity depends upon the manner in which arginine is linked in the protein molecule, a protein which contains a lesser amount of arginine than another protein may still give a more intense color.

(9) The Sullivan Reaction. This reaction makes use of 1,2-naphthoquinone-4-sodium sulfonate. Its capacity to react with certain other compounds with the production of complexes of high tinctorial power has been recognized for a long time. Folin (13) employed the compound for the determination of amino acids in blood. The reaction as employed by Sullivan (16) differs from all other methods in that the color development takes place in a strongly reducing atmosphere. Under these conditions, the reaction is remarkably specific for cysteine. In order to obtain a positive test, it is required that the three groups which are present in cysteine, —SH, —NH₂, —COOH, be free and in the same configuration as that which occurs in natural cysteine, since isocysteine does not respond to this reaction. The test, both as Sullivan described it and in various modifications, has been quite extensively used for the determination of cystine in protein hydrolysates and in urine (17). The reaction, when carried out exactly as described by Sullivan, is so specific that the only compounds which have been found to be exceptions are cystine dimethyl ester, cystinyldiglycine, and cystinyldialanine (18). The equivalent of one milligram of cystine in the form of a peptide gives a far more intense color than does one milligram of free cystine. This color is not the characteristic one which is obtained with cystine, but is magenta in appearance. Hence it is difficult, if not impossible, to determine cystine colorimetrically in the presence of any one of the above compounds. It might be appropriate to point out that certain substances which are analogues of cystine, for example, homocystine, not only fail to give the Sullivan reaction, but, if they are present, depress the color given by cystine.

2. GENERAL QUANTITATIVE METHODS FOR THE ESTIMATION OF THE AMINO ACIDS AND PROTEINS

A. Formol titration

Since the pH of an aqueous solution of the monoaminomonocarboxylic acids changes so slowly on titration that no sharp break in the titration curve occurs, titration with acids or alkalies in the presence of the usual indicators cannot be carried out. Sörensen (19), in 1907, found that when formaldehyde is added to an aqueous solution of these amino acids the titration can be carried out

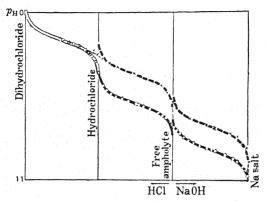


Fig. 1. Titration curves of histidine, in water and in HCHO.
O, in water. x, in HCHO (1%).
(Birch, T. W., and Harris, L. J., Biochem. J., 24, 1080, (1930).)

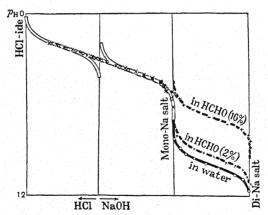


Fig. 2. Titration of aspartic acid in water and HCHO.

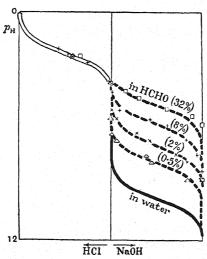
O, in water. x, in HCHO (2%). +, in HCHO (16%).

(Birch, T. W., and Harris, L. J., Biochem. J., 24, 1080, (1930).)

to a sharp end-point in the presence of phenolphthalein. This observation was based on an earlier one of Schiff (20), viz., that when formaldehyde is added to a solution of these amino acids, one equivalent of an amino acid is neutralized by one equivalent of strong alkali. Sörensen believed that the four components, water, amino acid, formaldehyde, and alkali, are in equilibrium. This is represented by the equation:

$HOOC \cdot R \cdot NH_2 + HCHO + O\overline{H} \rightleftharpoons \overline{O}OC \cdot R \cdot N : C : H_2 + 2H_2O$

The formol titration depends on the fact that there are more or less shifts in certain parts of the titration curves of the amino acids in the presence of formaldehyde. This is illustrated in Figs. 1 to 3. Harris (21) (22) preferred to think of the system as dependent on a compound of the amino acid and formaldehyde having a dissociation constant which differs from that of the original amino acid. We may consider that the values of the apparent acid dissociation



constants of the amino acids, in the presence of formaldehyde, differ from those which they have in aqueous solution. The apparent dissociation constants of a number of amino acids in water and in solutions of formaldehyde are given in Table I.

If the reaction takes place according to the concept which was postulated by Sörensen, it would appear that the carboxyl group of the amino acid is titrated by alkali in the presence of formaldehyde. On the basis of the Harris postulate and the zwitter ion concept of the structure of amino acids, the amino group is titrated as is indicated by the following equation:

$$\text{HCHO} + \bar{\text{O}}\text{OC} \cdot \text{R} \cdot \text{NH}_3 \rightarrow \bar{\text{O}}\text{OC} \cdot \text{R} \cdot \text{NH}_2 \cdot \text{CH}_2\text{O} + \text{H}^+$$

The question as to the number of molecules of formaldehyde which combine with a molecule of amino acid has arisen. Levy (23)

Table I
Summary of Data on Apparent Acid Dissociation Constants of Amino Acids in Water
and Aqueous Formaldehyde

	$pK_{a}{}^{\prime}$									
Amino acid*	Water†	16 per cent HCHO (Harris)	10 per cent HCHO (Levy);	9 per cent HCHO (Authors)						
dl-Alanine	9.68	6.4		6.96						
l-Aspartic acid	3.66	≦3.8								
	9.6	6.85								
d-Glutamic acid	4.25	≤ 4.2								
	9.66	6.8	6.83							
Glycine	9.60	5.4	5.61	5.92						
l-Leucine	9.60		6.83							
dl-Norleucine	9.77			7.10						
dl-Phenylalanine	9.12	5.9	6.53	6.80						
l-Proline	10.60		7.73							
dl-Serine	9.15			5.63						
l-Tyrosine	9.15	6.2	7.41	e e e e e e e e e e e e e e e e e e e						
	10.15	>9								
dl-Valine	9.64			7.47						

^{*} The optical forms of the amino acids as listed are assumed to be those which Harris and Levy used. The pK_a' value, 5.86, for β -phenyl- α -amino-propionic acid in 10 per cent formaldehyde was reported by Levy. However, it is not certain what compound was actually used, since this name appears to be erroneous.

† The $pK_{a'}$ values in water were calculated from what seemed to be the most reliable data in the literature. (See Chapter XI.)

Summary of Data on Apparent Acid Dissociation Constants of Peptides in Water and Aqueous Formaldehyde

	pK_{a}'						
Glycyl-dl-leucine. U-Alanylglycine. U-Leucylglycine. U-LeucylglycineLeucyl-l-tyrosine.	Water	9 per cent HCHO					
Glycylglycine	. 8.13	4.27					
Diglycylglycine		4.24					
Glycyl- dl -leucine		4.40					
dl-Alanylglycine	. 7.75	5.52					
dl-Leucylglycine		5.55					
		5.57					
l-Leucyl-l-tyrosine		5.07					
dl-Norleucylglycine		5.58					

The pK_{a} ' values in water were calculated from what seemed to be the most reliable data in the literature. The values in 9 per cent HCHO are the authors' experimental constants.

[‡] See also Levy, M., and Silberman, D. E., J. Biol. Chem., 118, 723 (1937).

⁽Dunn, M. S., and Loshakoff, A., J. Biol. Chem., 113, 691 (1936).)

Table I (Continued)

Effect of the Concentration of Formaldehyde Upon the Apparent Dissociation

Constants of Amino Acids

Coefficients of Apparent Acid Dissociation Constant Equations of Amino Acids*

Amino acid	a	b	k
Glycine	4.491	-3.330	26.52
dl-Alanine		-5.650	50.52
dl - α -Aminobutyric acid	5.763	-3.782	35.17
<i>dl</i> -Valine	5.717	-6.043	53.89
dl-Norvaline	5.664	-6.398	57.38
dl-Norleucine	5.399	-5.042	45.90
l-Norleucine	5.090	-6.483	58.09
dl-Leucine	5.430	-4.761	43.57
<i>l</i> -Leucine	5.421	-4.823	44.10
<i>dl</i> -Serine	4.437	-3.832	27.29
dl-Phenylalanine	5.658	-2.394	22.57

^{*} Apparent dissociation constant equations: xy - ax - by - k = 0, where x is mole per cent formaldehyde, y is pK_a' , and a, b, and k are numerical constants. (Dunn, M. S., and Weiner, J. G., J. Biol. Chem., 117, 381 (1937).)

has concluded that two molecules of formaldehyde combine with one molecule of amino acid forming an associated compound. On the other hand, Tomiyama (24) believes that a molecular compound is formed by means of the residual charge of both components or by coordinate valency and that, in the region of pH 8 to 10, one molecule of formaldehyde combines with one molecule of amino acid. He has used the following equation to calculate the change in pH in the alkaline region of the amino acid buffer when various amounts of formaldehyde are added, as well as the endpoint of the titration:

$$pH = -\log K_e + pK_a' + \log ([z^-]/[+A^-]) - \log [F - z^-]$$

 K_{ϵ} = equilibrium constant, K_{a}' = classical apparent acid dissociation constant, F = concentration of formaldehyde in moles, $+A^-$ = the zwitterion form of the amino acid, and z^- = the molecular compound which is formed. In order to calculate values for pH, the value of K_{ϵ} must be known.

From a theoretical standpoint the reactions which are involved in the formol titration are of great interest. For practical purposes it is immaterial whether, in the titration, one thinks in terms of the amino or the carboxyl group since either group can be calculated from the titration values. Practically, the formol titration has found extensive use and it is from that standpoint only that it will be considered further. According to Levy the maximum accuracy is

obtained when (a) the concentration of formaldehyde at the end of the titration is between 6 and 9 per cent, (b) neutral formalin is used, (c) no correction for a blank is made, (d) the concentration of amino acids is as high as possible, and (e) the end-point is in the vicinity of 9.1. It is probable that the latter value may depend on the amino acid which is being titrated.

Dunn and Loshakoff (25) have made use of the formol titration for purposes of determining the purity of preparations of amino acids. Using the glass electrode to determine the end-point of the titration they obtain an accuracy of ± 0.1 per cent.

The general procedure for the determination of amino acids, peptides, and proteins by means of the formol titration involves, first, the removal of coloring matter, ammonia, phosphates, and other substances which interfere with the titration; secondly, the addition of neutral formaldehyde; thirdly, titration with a standard solution of alkali, using phenolphthalein as indicator. Details of the technique have been described by Northrop (26).

The formol titration has been extensively used not only for the estimation of amino acids, peptides, and proteins in pure solutions and in biological fluids, but also to follow the course of protein hydrolysis.

B. Titration in the Presence of Other Solvents

The addition of certain non-aqueous solvents, such as alcohol and acetone, to an aqueous solution of amino acids or peptides permits an estimation of these substances by titrimetric methods. The principle which is involved depends on a shift in the end-point of the titration. The shift may be apparent, due to a change in the dissociation constant of the indicator (the pK' value of phenolphthalein is 8.6 in water and 12.6 in 90 per cent alcohol). On the other hand, the shift may be real, due to actual changes in the compounds which are being titrated, although this is probably not a primary effect of changes in the dielectric constant of the solvent (27).

Amino acids and their peptides exist predominantly in the zwitterion form in alcoholic as well as in aqueous solution. As has been shown by Jukes and Schmidt (27) (see Table II), the pK' value of a monoaminomonocarboxylic acid, representing the —COO group, is markedly increased by the addition of alcohol, while the

 pK_2' value which represents the —NH₃ group is changed but little. Hence the titration of amino acids with hydrochloric acid, as in the method of Van Slyke and Kirk (28), depends on a shift

Table II
Apparent Dissociation Constants

Substance	Valu	es in aqu solution		Values in 72 per cent ethanol solution			
	pK_1'	pK_{2}'	pK_3'	pK_{1}'	pK_2'	$pK_{\bf 3'}$	
Monoaminomonocarboxylic acids							
Alanine	2.36	9.72		3.55	10.02		
Asparagine	2.17	8.86		3.09	9.10		
Glycine	2.42	9.74		3.46	9.82		
Glycine	2.54	9.81		3.52	9.99		
Isoleucine	2.36	9.68		3.69	9.81		
Valine	2.32	9.62		3.60	9.73		
α -Amino- n -valeric acid	2.36	9.72		3.60	9.88		
γ -Amino- n -valeric acid	4.02	10.40		5.35	10.39		
δ -Amino- n -valeric acid	4.21	10.69		5.74	10.47		
Monoiminomonocarboxylic acid							
Proline	2.00	10.60		3.04	10.55		
Monoaminodicarboxylic acids							
Glutamic acid	2.19	4.25	9.66	3.16	5.63	10.75	
Aspartic acid	1.88	3.65	9.60	2.85	5.20	10.51	
Dibasicmonoacidic amino acids		100					
Arginine	2.02	9.04	12.48	3.34	9.40	14.1	
				3.07*			
Histidine	1.82	6.00	9.17	3.00	5.85	9.45	
Lysine	2.18	8.95	10.53	2.75	8.95	10.53	
				2.96	8.97	10.53*	
				3.23	9.00	10.53‡	
				3.56	8.95	10.49§	
Organic bases							
Ethylamine	10.82			10.20			
Cadaverine	9.84	10.98		9.49	10.32		
Glycine ethyl ester	7.73			7.19	a star		
Carboxy acids					4 1 1 1		
Acetic acid	4.70			6.41			
Monochloroacetic acid	2.86			4.44			

^{* 60} per cent ethanol solution.

in the pK' values of the —CO \overline{O} groups and the selection of a suitable indicator. A preliminary adjustment of the solution to pH 3.9 is made, and consequently the free basic groups (e.g., the basic group of lysine) are not titrated. Only the —CO \overline{O} groups which are in equilibrium with charged basic groups are titrated. In the cases of aspartic, glutamic, and β -hydroxyglutamic acids, a

^{† 48} per cent ethanol solution.

^{‡ 72} per cent ethanol solution.

^{§ 84} per cent ethanol solution.

⁽Jukes, T. H., and Schmidt, C. L. A., J. Biol. Chem., 105, 359 (1934).)

part of the second carboxyl group is titrated. If, on the other hand, the titration is carried out in alcoholic solution with a standard solution of alkali, the method depends on a shift in the pK' value of the indicator, and the charged basic groups are titrated in accordance with the chemical equation:

$$-NH_3+O\overline{H} \rightleftharpoons -NH_2+H_2O$$

Since the pK' of the indicator, rather than the pK' values of the basic groups, is increased by the addition of alcohol, both basic groups of lysine and of histidine are titrated with alkali when the pH of the solution is first adjusted to 3.9. In this procedure the predominantly basic groups of lysine and histidine are converted to salts by titration with acid to pH 3.9. The amount of acid which has been combined is determined by subsequent titration with alkali. The pK_3 value of arginine which corresponds to the highly basic resonant guanidine group (29), however, does not, even in alcoholic solution, fall within the range of the ordinary indicators.

- (1) The various procedures which are given below depend on the principles which have just been stated. In Foreman's (30) method the titration with a standardized solution of alkali is carried out in 85 per cent alcohol, using phenolphthalein as indicator. With the exception of aspartic, glutamic, β -hydroxyglutamic acids, proline, hydroxyproline, and arginine, all of the amino acids can be determined by this procedure. On addition of formaldehyde to the solution, the other amino acids, except arginine, can be determined. The method is not specific for amino acids since ammonium salts and organic acids, if present, are likewise titrated. Hence the attempted application of this method to the estimation of amino acids and peptides in biological fluids must be carefully interpreted.
- (2) In the methods proposed by Willstätter and Waldschmidt-Leitz (31) two different strengths of alcohol are used. An alcoholic, instead of an aqueous solution of potassium hydroxide, is used and phenolphthalein is replaced by thymolphthalein. The authors claim that polypeptides can be titrated in 40 per cent alcohol. By titrating first in 40 per cent alcohol and subsequently increasing the concentration of alcohol to 97 per cent, both polypeptides and amino acids can be estimated in the same solution. The method has the same disadvantages as the Foreman method. It has the further disadvantage that, when solutions of proteins or partially hydrolyzed proteins are titrated, the high concentrations of alcohol produce precipitates which obscure the end-point.

(3) In Harris' procedure (21) the titration is carried out in 85 per cent alcohol, using thymolphthalein as indicator, until a blue color appears. Methyl red is then added and the solution is titrated with 0.1 N hydrochloric acid to an orange color. Aside from the general considerations which apply to titrations in non-aqueous solvents, there are the added disadvantages such as the uncertainty of the second end-point, especially when precipitates are

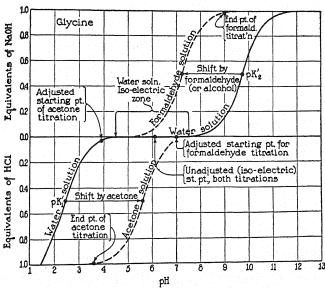


Fig. 4. Titration curve of glycine. Water solution data from Harris (1923-24). Formaldehyde curve estimated from end-points at pH 9 to 10 given by Sörensen (19). Acetone curve estimated from titration results of Linderström-Lang (33). (Van Slyke, D. D., and Kirk, E., J. Biol. Chem., 102, 651 (1933).)

produced, and the considerable dilution of the solution (32) which results from the addition of alcohol and solutions of acid and alkali. The general considerations which are involved in titrations in non-aqueous solvents apply to procedures in which acetone is used. Naphthyl red (benzene-azo- α -naphthylamine) is usually used as indicator when the titration is carried out in the presence of acetone (33). The comparative effects of acetone and of formaldehyde on the titration curves of certain amino acids are shown in Figs. 4 to 6.

(4) Amino acids may also be titrated in glacial acetic acid solution (34). The titration is carried out with a standardized solution of sulfuric, hydrobromic, or perchloric acid, using crystal violet, α -naphtholbenzein, or benzoyl auramine as indicator. It is cus-

tomary to titrate 0.1 to 0.2 gm. of amino acid in about 30 cc. of glacial acetic acid. Arginine and lysine are diacidic bases in acetic acid solution, while only one of the nitrogen atoms of tryptophane is estimated. The presence of picric acid does not interfere with the titration when the picric acid salt of the amino acid is titrated. In

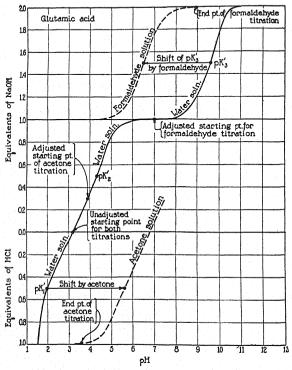


Fig. 5. Titration curve of glutamic acid. Water solution data from Kirk and Schmidt (1929). Formaldehyde curve estimated from end-points of Sörensen. Acetone curve estimated from titration results of Linderström-Lang.

(Van Slyke, D. D., and Kirk, E., J. Biol. Chem., 102, 651 (1933).)

¹ (Kirk, P. L., and Schmidt, C. L. A., Univ. of Calif. Pub. Physiol., 7, 57 (1929).)

the case of amino acids which are insoluble in glacial acetic acid, the titration may be carried out by dissolving the amino acid in 0.1 N perchloric acid and back-titrating with guanidine acetate.

C. The Gasometric Estimation of Amino Acids

Van Slyke (35) has described a method whereby amino acids can be determined quantitatively by treating them with nitrous acid and estimating the amount of nitrogen gas which is evolved. The reaction is:

 $R \cdot CH(NH_2) \cdot COOH + HNO_2 \rightarrow R \cdot CH(OH) \cdot COOH + N_2 + H_2O$

The apparatus which is used is illustrated in Fig. 9. An amount of glacial acetic acid, equal to about one-fifth of the volume of the reaction chamber, is introduced. This is followed by the addition of a 30 per cent sodium nitrite solution. The mixture is shaken several times and the gas which is evolved is permitted to escape in

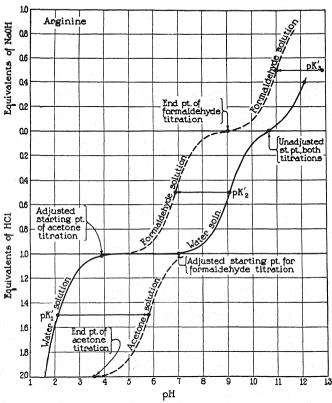


Fig. 6. Titration curve of arginine. Water solution data from Hunter and Borsook (1924). Formaldehyde curve estimated from end-point of Sörensen (19). Acetone curve estimated from titration results of Linderström-Lang (33).

(Van Slyke, D. D., and Kirk, E., J. Biol. Chem., 102, 651 (1933).) ¹ (Hunter, A., and Borsook, H., Biochem. J., 18, 883, (1924).)

order to free the reaction chamber of any traces of air. In the final shaking about one-half of the mixture in the reaction chamber is displaced by the evolved gas. The solution of amino acid, peptide, or protein is now introduced into the reaction chamber through the side burette, and the mixture is shaken for a period of about 4 minutes. During this time the gases are collected in the gas burette. At the end of this time all of the gas is transferred to the gas burette. In addition to nitrogen the gas contains nitric oxide which

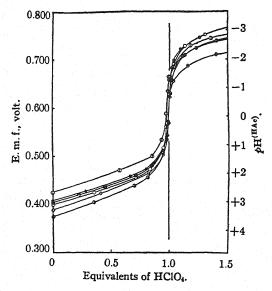


Fig. 7. Titration curves of amino acids soluble in acetic acid.

•, Glycine; \odot , α -amino-n-valeric acid; \ominus , dl- β -phenylalanine;

•, d-lysine picrate; half shade circle, l-proline; \oplus , l-tryptophane.

(Nadeau, G. F., and Branchen, L. E., J. Amer. Chem. Soc., 57, 1363 (1935).)

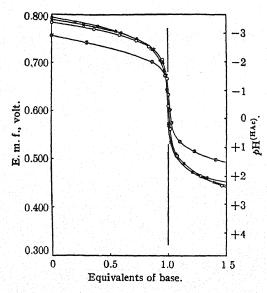


Fig. 8. Titration curves of amino acids that are insoluble in glacial acetic acid. The amino acid was dissolved by addition of 0.1 N perchloric acid and backtitrated with guanidine acetate.

○, l-aspartic acid; ●, d-glutamic acid;
 ①, l-cystine; ×, l-tyrosine.
 (Nadeau, G. F., and Branchen, L. E., J. Amer. Chem. Soc., 57, 1363 (1935).)

results from the decomposition of the nitrous acid as is shown by the reaction:

The nitric oxide gas is absorbed by passing the gases into an alkaline solution of potassium permanganate. The nitrogen is then measured in the gas burette and is reduced to standard conditions by means of the factors which are given in Table III. When the barometric pressures are greater than those which are given in

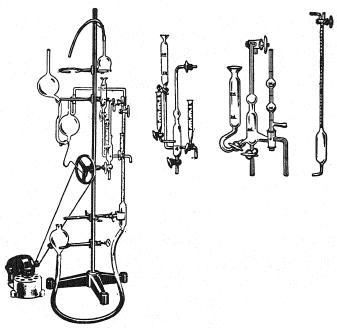


Fig. 9. Van Slyke amino nitrogen apparatus. (See also Kendrick, A. B., and Hanke, M. E., J. Biol. Chem., 117, 161 (1937).)

Table III, the factor F (mgs. of nitrogen which correspond to 1 cc. of gas) may be calculated with the aid of the equation (36). A correction for any unabsorbed gases which are given off by the reagents is determined by carrying out a blank determination in which water instead of a test solution is used.

$$F = \frac{(\text{Bar.} - \text{vapor press. H}_2\text{O at } t^\circ) \left(\frac{0.96727 \times 0.0012931}{2}\right)}{760[1 + (0.003675t)]} \times 1000$$

Details of the technique for estimating amino nitrogen are given by Morrow (37).

On account of the high degree of accuracy, as well as its speed, the method has proved of great value for the estimation of amino

TABLE III

Milligrams of Amino Nitrogen Corresponding to 1 cc of Nitrogen Gas at 11°-30°C.;

728-778 mm. Pressure

t	728	730	732	734	736	738	740	742	744	746	748	750	t
11°	0.5680	0.5695	0.5710	0.5725	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5840	0.5855	119
12°	0.5655												129
13°	0.5630												139
14°	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5700	0.5715	0.5730	0.5745	0.5760	0.5775	14
15°	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5705	0.5720	0.5735	0.5750	15
16°	0.5555	0.5570	0.5585	0.5600	0.5615	0.5630	0.5645	0.5660	0.5675	0.5690	0.5710	0.5725	16
17°												0.5695	17
18°	0.5500	0.5515	0.5530	0.5545	0.5560	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	18
19°	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5630	0.5645	19
20°	0.5445	0.5460	0.5475	0,5495	0.5510	0.5525	0.5540	0.5555	0.5570	0.5585	0.5600	0.5615	20
21°	0.5420	0.5435	0.5450	0.5465	0.5480	0.5495	0.5510	0.5525	0.5540	0.5555	0.5575	0.5590	21
22°	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	0.5545	0.5560	22
23°	0.5365	0.5380	0.5395	0.5410	0.5425	0.5440	0.5455	0.5470	0.5485	0.5500	0.5515	0.5530	23
24°	0.5335	0.5350	0.5365	0.5380	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	24
25°	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	25
26°	0.5260	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5365	0.5400	0.5415	0.5430	0.5445	26
270	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	0.5400	0.5415	27
28°	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	0.5370	0.5385	28
29°	0.5195	0.5210	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	0.5340	0.5355	29
30°	0.5160	0.5175	0.5190	0.5205	0.5220	0.5235	0.5250	0.5265	0.5280	0.5295	0.5310	0.5325	30
t	728	730	732	734	736	738	740	742	744	746	748	750	t

t	752	754	756	758	760	762	764	766	768	770	772	t
11°	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	0.5965	0.5980	0.5995	0.6010	0.6030	11°
12°	0.5845	0.5860	0.5875	0.5890	0.5905	0.5925	0.5940	0.5955	0.5970	0.5985	0.6000	12°
13°	0.5820	0.5835	0.5850	0.5865	0.5880	0.5895	0.5910	0.5930	0.5945	0.5960	0.5975	13°
14°	0.5790	0.5805	0.5825	0.5840	0.5855	0.5870	0.5885	0.5900	0.5915	0.5935	0.5950	14°
15°	0.5765	0.5765	0.5795	0.5810	0.5830	0.5845	0.5860	0.5875	0.5890	0.5905	0.5920	15°
16°	0.5740	0.5755	0.5770	0.5785	0.5800	0.5815	0.5830	0.5850	0.5865	0.5880	0.5895	169
17°	0.5710	0.5730	0.5745	0.5760	0.5775	0.5790	0.5805	0.5820	0.5825	0.5850	0.5865	179
18°	0.5685	0.5700	0.5715	0.5730	0.5745	0.5765	0.5780	0.5795	0.5810	0.5825	0.5840	189
19°	0.5660	0.5675	0.5690	0.5705	0.5720	0.5735	0.5750	0.5765	0.5780	0.5795	0.5810	199
20°	0.5630	0.5645	0.5660	0.5675	0.5690	0.5705	0.5725	0.5740	0.5755	0.5770	0.5785	20
21°	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	0.5710	0.5725	0.5740	0.5755	21
22°	0.5575	0.5590	0.5605	0.5620	0.5635	0.5650	0.5665	0.5680	0.5695	0.5715	0.5730	229
23°	0.5545	0.5560	0.5575	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	0.5685	0.5700	239
24°	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	0.5655	0.5670	249
25°	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	0.5625	0.5640	25
26°	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	0.5595	0.5610	26
27°	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	0.5535	0.5550	0.5565	0.5580	279
28°											0.5550	289
29°	0.5370	0.5385	0.5400	0.5415	0.5430	0.5445	0.5460	0.5475	0.5490	0.5505	0.5520	299
30°											0.5490	30
t	752	754	756	758	760	762	764	766	768	770	772	•

(Van Slyke, D. D., J. Biol. Chem., 12, 275 (1912).)

nitrogen in amino nitrogen-containing compounds both in pure solution and in biological fluids. The course of the hydrolysis of proteins may be estimated by determining the increase, with time, in amino nitrogen in the hydrolysate. Small quantities of material can be used in the determination. With the micro-apparatus, 0.5 mg. of amino nitrogen can be determined with an accuracy of about 1 per cent.

Although the Van Slyke procedure permits the quantitative determination of α -amino groups, certain exceptions must be borne in mind. The α -amino group reacts quantitatively with nitrous acid at room temperatures. The nitrogen from the e-amino group of lysine is usually liberated quantitatively in a period of 30 minutes. Thus when lysine is treated with nitrous acid for a period of 4 minutes, all of the α -amino nitrogen and a part of the ϵ -amino nitrogen will be set free. The time which is required for the amino group to react with nitrous acid at 23° increases directly as the amino group is shifted from the α - to the ϵ -position in the molecule (38). Cystine and glycine, due to oxidation, yield an excess over the theoretical amount of amino nitrogen which is present in the molecule, even when the time of the reaction is short. At 45°, arginine, tryptophane, histidine, and serine likewise yield high values (39). Kendrick and Hanke (40) have proposed the use of potassium iodide in acetic acid in order to obtain theoretical values for glycine, cystine, leucine, alanine, tyrosine, and glutamic acid. The entire analysis may be carried out in the Harington-Van Slyke (75) chamber, thus avoiding the use of the Hempel pipette.

About 6 hours are required for the guanidine group of arginine to give off an amount of nitrogen which is equivalent to one-half of the total nitrogen content of arginine (41). Many other compounds, such as urea, asparagine, the purine compounds, and creatinine, also react with nitrous acid (42).

D. Specific Quantitative Estimation of Individual Amino Acids

(1) Hausmann Numbers. In 1899 Hausmann (43) proposed a method for the quantitative differentiation of proteins according to their hydrolytic products. The nitrogen is distributed into three different groups, viz., the ammonia or amide nitrogen, the diamino or basic nitrogen, and the monoamino or non-basic nitrogen. A fourth fraction, viz., humin or melanin nitrogen, was added later. The method consists in hydrolyzing the protein with a strong acid such as hydrochloric acid and then determining: (a) the humin

nitrogen (the insoluble black precipitate is filtered off and analyzed for total nitrogen; (b) the amide or ammonia nitrogen (when the solution is distilled with an excess of magnesia, the ammonia which is given off is determined); (c) the basic nitrogen (this represents the nitrogen of amino acids which are precipitated from the solution by means of phosphotungstic acid after having freed it from ammonia); (d) the nitrogen which remains in the filtrate after precipitation with phosphotungstic acid (this nitrogen includes that of the monoamino acids).

- (2) The Humin Nitrogen (44). The amount of nitrogen in this fraction is quite constant for a specific protein but varies from zero to approximately 10 per cent in different proteins. The variation in this fraction of nitrogen has already been discussed in Chapter III.
- (3) The Ammonia or Amide Nitrogen. After the removal of the humin nitrogen the excess of hydrochloric acid is removed by vacuum distillation and the solution is made up to a definite volume. An aliquot of this solution is then rendered alkaline with an excess or either magnesia, barium carbonate, or a suspension of calcium hydroxide, and the ammonia is distilled from the solution into standard acid, preferably in a partial vacuum. The ammonia which is formed is considered to be and has been quite definitely established as representing the amide nitrogen of the protein molecule. Since it has been shown that the amount of ammonia nitrogen parallels the total amount of aspartic, glutamic, and β -hydroxy-glutamic acids which are present in the protein molecule, it is considered that the amount of ammonia which is thus determined is a quantitative measure of these amino acids.

Many objections have been raised as to the accuracy of this method. It has been maintained, first, that the amount of amide nitrogen varies according to the strength of acid used; second, that it varies according to the length of time of hydrolysis; third, that some of the ammonia is precipitated with phosphotungstic acid in the subsequent procedure, and hence it was not completely removed by distillation; and, finally, that by distillation, even with the weak alkalies such as those which have been mentioned, a small amount of decomposition of some of the amino acids, for example arginine and cystine, takes place. These objections, however, are not very serious since the error which results from any of the above mentioned factors is quite small. When a three gram sample of fibrin was hydrolyzed with hydrochloric acid, 34.5 mgs. of ammonia nitrogen were obtained at the end of one hour; at the end

of 4 hours, 34.7 mgs.; a 12 hour hydrolysis gave 37.5 mgs.; a 24 hour hydrolysis, 40.6 mgs.; a 72 hour hydrolysis, 51.0 mgs.; a 201 hour hydrolysis, 69.3 mgs.; and a six weeks hydrolysis, 100.3 mgs. of ammonia nitrogen. Most hydrolyses are complete within 24 hours. The figures which are cited above show that no great error in the estimation of amide nitrogen results by varying somewhat the time of hydrolysis. It is interesting to note that it has been found that the deamination which takes place during hydrolysis involves only the monoamino acids. Arginine, histidine, and lysine are not deaminized by boiling with strong hydrochloric acid for a period of 6 weeks.

- (4) The Basic Nitrogen. The basic nitrogen is determined by precipitating arginine, histidine, lysine, and cystine by means of phosphotungstic acid in the presence of an excess of hydrochloric acid. Under certain conditions the phosphotungstates of these amino acids are relatively insoluble. The precipitate is filtered. washed with a solution containing phosphotungstic acid and hydrochloric acid, and the total nitrogen of the entire precipitate is determined by means of the Kjeldahl method. The criticisms that have been made and which are valid for the interpretation of the results obtained by this method are primarily two: first, the phosphotungstates of the bases are relatively soluble, hence the volume of solution from which they are precipitated and the volume of the wash water must be kept as small as possible and ice cold; and second, other substances are precipitated along with the bases by means of phosphotungstic acid. For example, small amounts of tyrosine, tryptophane, and phenylalanine have been found in the phosphotungstate precipitate. By carefully controlling the conditions under which the precipitation is carried out, this method can be used for the separation of the basic from the monoamino acids of protein hydrolysates with a high degree of accuracy.
- (5) The Non-Basic or Monoamino Fraction. The total nitrogen of this fraction is determined on the filtrate from the phosphotung-state precipitate. The nitrogen of this fraction represents the monoaminomonocarboxylic acids and the monoamino-dicarboxylic acids. It is evident that the Hausmann method cannot be used for extremely accurate determinations. However, by careful control of the conditions, it is possible to obtain valuable comparative results, and marked differences between certain proteins can be made plainly evident. The advantages of the method are its

rapidity and the small amount of protein which is required for the determination. The method yields rather definite information as to the ratio between the monoamino acids and the diamino acids in proteins. A disadvantage which has not already been mentioned is that the individual amino acids are not determined in this method.

(6) The Van Slyke Nitrogen Distribution Method. In most of the methods which were available in 1911, considerable amounts of protein were required. The data which were obtained on the nitrogen distribution in proteins were not very definite, neither were the values quantitative. In order to overcome these disadvantages in part, Van Slyke (45) at this time proposed his well-known nitrogen distribution method. The method is a departure from the previous methods of Kossel and Kutscher (see next section), and of Fischer (see Chapter III), in that the amino acids, instead of being determined directly, are calculated from a determination of the groups which characterize them. The method involves the removal of the ammonia by vacuum distillation and the precipitation of the arginine, histidine, lysine, and cystine with phosphotungstic acid. The precipitate is redissolved and these four amino acids are determined on the basis of their marked chemical differences.

Cystine is determined on the basis of the sulfur content. The difference between the total nitrogen content and the amino nitrogen content of the basic amino acids gives the non-amino nitrogen of these acids. This results from the fact that, when treated with nitrous acid for 30 minutes, lysine gives off all, arginine one-fourth, and histidine one-third of its content of nitrogen. Arginine is determined by boiling an aliquot portion of the solution with concentrated alkali whereby one-half of its total content of nitrogen is evolved as ammonia. A convenient apparatus for carrying out this procedure has been described by Holm (46). The amount of histidine is determined as follows:

Histidine
$$N=3/2$$
 (Non-amino nitrogen of the basic acids—3/4 arginine nitrogen). (1)

or,

Histidine N=1.5 Non-amino nitrogen of the basic acids—1.125 arginine nitrogen.

The amount of lysine is calculated by difference:

Nitrogen of lysine = Total nitrogen—(nitrogen of arginine+cystine+histidine).

(2)

The amino acids which are present in the filtrate after precipitation of arginine, cystine, histidine, and lysine are divided into two sub-groups: (a) the amino acids which yield all of their content of nitrogen when treated with nitrous acid (alanine, aspartic acid, glycine, etc.); (b) the amino acids which do not yield free nitrogen (proline and hydroxyproline) or only a part (one-half of the content of nitrogen in tryptophane) of their total content of nitrogen when treated with nitrous acid. The latter procedure divides the nitrogen into (a) amino and (b) non-amino nitrogen. Tryptophane yields one-half of its nitrogen to each of these groups.

The distribution of nitrogen in the Van Slyke procedure, as modified, may be schematically represented as follows:

- Total nitrogen of protein.
 (Estimate nitrogen in a sample of protein by Kjeldahl method.)
- 2. Hydrolysis of protein.

 (Boil protein with conc. HCl until there is no increase in amino nitrogen.)
- 3. Acid insoluble humin nitrogen.

 (Filter solution and wash precipitate with water. Estimate nitrogen by Kjeldahl method.)
- 4. Amide (ammonia) nitrogen.
 - (Remove excess of HCl in filtrate from procedure 3 by vacuum distillation. Add excess of Ca(OH)₂ suspension. Distill ammonia under diminished pressure.)
- Acid soluble humin nitrogen.
 (Filter residue from procedure 4. Wash precipitate until free from chlorides. Estimate nitrogen in residue by Kjeldahl method.)
- 6. Precipitation by means of phosphotungstic acid.
 - (Acidify filtrate from procedure 5, concentrate in vacuo, add conc. HCl and phosphotungstic acid, filter precipitate, and wash it with a HCl-phosphotungstic acid solution until free from calcium.)

The amino acids are now divided into 2 fractions: (a) precipitate contains arginine, cystine, histidine, lysine; (b) filtrate contains other amino acids.

7. Phosphotungstic acid humin nitrogen.
(Decompose phosphotungstate precipitate with NaOH solution. Precipitate phosphotungstic acid with BaCl₂. Filter

precipitate and wash free from chlorides. Estimate nitrogen in precipitate by Kjeldahl method. Acidify filtrate with HCl.)

8. Total and amino nitrogen of amino acids which are precipitated by phosphotungstic acid.

(Determine total nitrogen in an aliquot portion of the filtrate from procedure 7 by Kjeldahl method. Estimate amino nitrogen in an aliquot portion by treating with HNO₂ for 30 minutes.)

9. Distribution of nitrogen in the fraction which is precipitated by phosphotungstic acid.

(a) Cystine.

(Estimate sulfur content in an aliquot portion of the filtrate from procedure 7.)

(b) Arginine.

(Add an excess of alkali to an aliquot portion of the filtrate from procedure 7. Distill ammonia into a standardized solution of acid. Calculate the amount of arginine from the value of the ammonia nitrogen.)

(c) Histidine.

(Calculate by means of equation (1).)

(d) Lysine.

(Calculate by means of equation (2).)

10. Distribution of nitrogen in amino acids which are not precipitated by phosphotungstic acid.

(Estimate the total and the amino nitrogen in the solution obtained from procedure 6b.)

(a) Amino nitrogen.

(Nitrogen from alanine, glycine, etc., and $\frac{1}{2}$ of the nitrogen of tryptophane.)

(b) Non-amino nitrogen.

(Total nitrogen—amino nitrogen obtained from above procedure. This represents the nitrogen of proline, hydroxy-proline, and $\frac{1}{2}$ of the nitrogen of tryptophane.)

Details of the above technique are given by Morrow (37). In carrying out an analysis by the above procedure, it is necessary that the protein be free from nitrogenous impurities such as the purine bases and ammonia.

Many criticisms of the Van Slyke method have been made. These have dealt chiefly with the accuracy with which the various fractions may be determined. However, the Van Slyke method should not be severely criticized from this angle. The fault lies with the interpretations which certain workers have placed on the values. Van Slyke himself has often stated that he never claimed the accuracy for the method which many persons have attempted to assign to it. The errors and limitations of the method which have been recognized by Van Slyke and by other investigators are the following:

- (a) Errors due to hydrolysis. Cystine, proline, and tryptophane are, in part, decomposed during the acid hydrolysis of the protein. The products which are formed will influence the values for amino and total nitrogen in certain of the fractions which are given in the above outline. The value for cystine may be as much as 50 per cent in error.
- (b) Errors in the estimation of amino nitrogen. Cystine and glycine yield more than the theoretical amounts of nitrogen unless the method of Kendrick and Hanke (40) is used. Decomposition of tryptophane during hydrolysis will influence the value for amino nitrogen of the non-basic nitrogen fraction. When the basic amino acids are treated with nitrous acid for a period of 30 minutes, some of the nitrogen from the guanidine group of arginine will be liberated. This will influence the values for histidine and lysine.
- (c) Errors due to precipitation. The precipitation of arginine, cystine, histidine, and lysine by phosphotungstic acid is not wholly quantitative. Certain of the other amino acids may be partially precipitated by phosphotungstic acid.
- (d) Other errors. If the amount of cystine which is present in the protein is large, the value for arginine will be affected since, in the alkaline decomposition of arginine, as much as 20 per cent of the nitrogen of cystine will be converted into ammonia. In the calculation of lysine, nitrogen errors which result in the estimation of arginine, cystine, and histidine are included.

The Van Slyke distribution method has nevertheless been very useful in the characterization of proteins. Its limitations should be clearly recognized and values should not be reported beyond the limits of accuracy. In comparing the Van Slyke method with that of Kossel and Patten (47), it is generally true that, in the case of lysine, the Van Slyke method is the more accurate. The Kossel and Patten technique yields more consistent results for histidine. Both methods give fairly reliable values for arginine.

- (7) The Fischer Esterification Method. This method has been discussed in detail in Chapter III. The method is applicable, as previously pointed out, primarily to the determination of those amino acids whose esters can be distilled under reduced pressures. It is of great value in the determination of certain of the amino acids which do not form special compounds by which they may be identified or have no characteristic color reactions. The method is not extensively used for either qualitative or quantitative determinations.
- (8) The Kossel and Kutscher Silver-Baryta Method. One of the oldest and best methods for the estimation of the basic amino acids which are obtained on hydrolysis of proteins is the silver-baryta method which was described by Kossel and Kutscher in 1900 (48). It depends on the formation of insoluble silver compounds of arginine and histidine in the presence of fixed alkalies. These compounds had been described by Kossel in 1898. The method can be divided into the following parts:
 - 1. Hydrolysis of the protein with sulfuric acid.
 - 2. Removal of sulfuric acid.
 - 3. Precipitation of arginine and histidine.
 - 4. Separation of histidine and arginine. Estimation of histidine.
 - 5. Estimation of arginine.
 - 6. Estimation of lysine.

The hydrolysis of the protein is best carried out by means of boiling sulfuric acid in a concentration of 33 per cent by volume and for a period of approximately 20 hours. Five-tenths to five grams of protein are required in the estimation. The quantity depends on the content of basic amino acids. The amount of sulfuric acid which is used is about ten times the weight of the protein used. When the hydrolysis is complete, the solution is made up to a definite volume and an aliquot is taken for the determination of total nitrogen. Most of the sulfuric acid is subsequently removed by means of barium hydroxide. The resulting barium sulfate is thoroughly washed. The nitrogen which cannot be removed from the barium sulfate is calculated by estimation of the total nitrogen of the filtrate and subtraction from the previous total nitrogen value. The arginine and histidine are separated as the silver compounds by treating the solution with a boiling solution of silver sulfate and stirring constantly. The end-point of the reaction is determined by transferring a drop of the solution on a glass rod to a drop of baryta on a watch glass. If the precipitate formed is brown, sufficient reagent has been added. When the reaction is complete the precipitate which is formed is filtered off and washed with a dilute solution of barium hydroxide. The precipitate contains the silver salts of arginine and histidine, while the lysine and monoamino acids remain in solution.

In order to separate the arginine and histidine, the precipitate is suspended in dilute sulfuric acid and the silver is removed with hydrogen sulfide. The filtrate and washings are boiled to remove hydrogen sulfide and subsequently are made up to a definite volume. The total nitrogen is then determined. The separation of the arginine and histidine depends on the fact that the histidine-silver compound is precipitated in a less alkaline solution than is arginine. In order to carry out this separation, excess of silver nitrate and nitric acid are added to the filtrate. The solution is then gradually made alkaline with barium hydroxide until a point is reached where the histidine is completely precipitated. An easy way to do this is to add barium carbonate to the neutral solution of the silver compounds and then heat the solution. The alkalinity produced by the arginine carbonate is sufficient to cause the precipitation of the silver compound of histidine without any separation of arginine. The precipitate contains the histidine. After filtration it is suspended in sulfuric acid and the silver is removed with hydrogen sulfide. The nitrogen content of the solution gives a measure of the histidine which is present in the original protein.

The arginine is estimated in the filtrate from the histidine-silver compound. An estimation of the total nitrogen in this solution gives a means of calculating the amount of arginine.

Kossel and Staudt (49) have described a method for the estimation of arginine by precipitating it with flavianic acid. The difference between the nitrogen content of the silver nitrate-barium hydroxide precipitate and the nitrogen content of arginine flavianate gives the histidine nitrogen.

Lysine is estimated in the filtrate from the silver salts of arginine and histidine by removing the silver and precipitating the lysine as the phosphotungstate. After removal of the phosphotungstic acid a total nitrogen determination gives an estimate of the amount of lysine which is present in the original protein. The lysine can also be precipitated by picric acid and estimated gravimetrically. By means of this method the following fractions of nitrogen are determined:

- A. Total nitrogen of the protein.
- B. Nitrogen in the filtrate from the first barium sulfate precipitate.
- A-B. Nitrogen adsorbed by the barium sulfate precipitate.
 - C. Nitrogen in the fraction precipitated by silver and baryta (arginine+histidine).
 - D. Histidine nitrogen.
 - E. Arginine nitrogen.
 - F. Nitrogen of lysine+monoamino acids.
- B-(C+F). Nitrogen adsorbed by silver sulfide.
 - G. Monoamino-acid nitrogen.
 - F-G. Lysine nitrogen.

Several modifications of the Kossel and Kutscher method have recently been described. The most applicable modification and the most extensive study of the method have been made by Vickery and his co-workers (50). Their chief contribution has been the accurate control of hydrogen ion concentration in the procedures and the quantitative isolation of well characterized salts of the three basic amino acids. In the most recent modification (50) the hydrolysis is carried out with strong hydrochloric acid. Most of the hydrochloric acid is removed by distillation in vacuo, and the remainder is precipitated with silver nitrate in a strongly acid solution (pH less than 3). Excess of silver nitrate is added and the solution is made alkaline to pH 11 to 12 in order to precipitate the arginine and histidine as the silver salts. From this point the procedure is practically the same as that of Kossel and Kutscher except that the histidine is precipitated at exactly pH 7.2. Cystine is removed from histidine by treatment with copper hydroxide. After being certain that excess of silver is present, arginine is precipitated by the addition of hot barium hydroxide solution until the mixture is strongly alkaline to alizarin yellow R. After obtaining the histidine and arginine as the silver salts, and the lysine as the phosphotungstate, these amino acids are estimated individually by isolating arginine as the mono-flavianate, histidine as the diflavianate, and lysine as the picrate.

E. Some Special Methods for the Estimation of Individual Amino Acids

Besides the general methods for the estimation of amino acids, a considerable number of methods for the estimation of individual amino acids in proteins have been proposed. Reference to Sullivan's method for the determination of cystine and the Sakaguchi method for estimating arginine has already been made. It is not the purpose here to enumerate all of these methods nor to describe them in detail. Many of the methods are given by Mitchell and Hamilton (1). The more recent methods are listed in the Annual Review of Biochemistry (11). Those who desire to make use of the analytical methods should consult the original articles. The more important of the special methods are listed below. In using any of these methods the possibility that an amino acid other than the one which is to be estimated may give the reaction should always be borne in mind unless there is very definite evidence to the contrary. If interfering amino acids must first be removed, there is the possibility that the separation may not be quantitative.

(1) Tyrosine. The determination of tyrosine is based chiefly on colorimetric reactions. The method of Folin and Looney (53) depends on the blue color which phenolic compounds give with a phosphotungstic-phosphomolybdic reagent. After hydrolysis of the protein, tryptophane is removed with mercuric sulfate. The precipitation of tyrosine is prevented by addition of sulfuric acid to a concentration of 3.5 to 7.5 per cent. The values which have been reported are higher than those which have been obtained by isolating tyrosine. This is to be expected since isolation methods are rarely quantitative. The reagent of Folin and Looney is not specific for tyrosine.

The method of Folin and Ciocalteu (54) makes use of Millon's reagent. Tyrosine ordinarily reacts very slowly in the cold with this reagent. After tyrosine has been boiled with mercuric sulfate, it now reacts almost immediately. Equally good results can be obtained by substituting sodium nitrite for Millon's reagent. The color which is obtained is compared with the color which is given by a known solution of tyrosine.

Hanke and Koessler (55) have made use of the color which phenols give in alkaline solution when treated with diazotized sulfanilic acid for purposes of estimating tyrosine. The color is stabilized by the addition of sodium hydroxide, followed by a small amount of hydroxylamine hydrochloride. Since the reagent is not specific for tyrosine, it is necessary that histidine be removed as the silver salt after which tyrosine is precipitated as the mercury salt by the addition of mercuric acetate and sodium chloride. After removing the mercury, tyrosine is estimated colorimetrically.

The determination of tyrosine by bromination (56) has not found extensive use. Certain other amino acids also absorb bromine.

(2) Cystine. The naphthoquinone method of Sullivan (16) has already been described. Rossouw and Wilken-Jorden (57) determine cystine by precipitating it as cysteine cuprous mercaptide after which the modified Sullivan method is applied. Okuda's (58) method makes use of the fact that cystine can be reduced to cysteine. This is subsequently titrated with a solution of potassium bromate in the presence of potassium iodide. In a modification of the method. Okuda states that cystine can be determined in the presence of tyrosine, tryptophane, and other amino acids. This method has been adapted to the estimation of cystine in urine by Virtue and Lewis (17). Urinary cystine may also be determined by the method of Sullivan and Hess (74). A method for titrating cysteine iodometrically has been described by Lavine (59). Folin and Looney (53) use the blue color which is given by cystine when it is treated with phosphotungstic acid in the presence of sodium sulfite. The method is used but little.

Baernstein (60) finds that hydrolysis of proteins with hydriodic acid is more rapid than it is when hydrochloric acid is used. No humin is formed, cystine is reduced to cysteine, methionine is demethylated, and sulfates are reduced to hydrogen sulfide. Cysteine is determined titrimetrically by the method of Okuda. The accuracy of the method is about 97 per cent.

Since other sulfur-containing amino acids are present, methods which are based on the sulfur content of proteins or on feeding experiments are not specific for cystine. The estimation of cystine by isolation is not quantitative. Possible loss of cystine during hydrolysis should always be borne in mind when this amino acid is to be estimated.

(3) Tryptophane. Since tryptophane is destroyed by acid hydrolysis, it is necessary to hydrolyze the protein by means of either enzymes or alkali. Among the more recent methods for the estimation of tryptophane is that of Folin and Ciocalteu (54). This amino acid is precipitated with mercuric sulfate and, after removal of the mercury, the phenol reagent of Folin and Looney (53) is added. The resulting blue color is matched against the color which a known solution of tryptophane gives with this reagent. Tomiyama and Shigematsu (61) have reported the conditions which must be observed so that tryptophane will give a maximum blue color when it is treated with p-dimethylaminobenzaldehyde dissolved in 10

per cent sulfuric acid (Ehrlich's reagent). The color is compared against the blue color which is given by a solution of potassium acid phosphate after treatment with ammonium molybdate and eikonogen. The latter color is standardized in terms of tryptophane. The results which have been obtained by the use of this method compare favorably with those which have been reported by Folin and Ciocalteu (54).

Most of the other methods which have been proposed have not found general use. The literature is cited by Mitchell and Hamilton (1).

- (4) Histidine. This amino acid is usually determined by the methods which have been described for the estimation of the basic amino acids. Koessler and Hanke's (62) method makes use of the color which histidine gives when it is treated with p-diazobenzene sulfonate. Histidine is precipitated from the protein hydrolysate with silver nitrate and barium hydroxide, the silver is removed by addition of hydrochloric acid, and the histidine in the filtrate is determined colorimetrically. Kapeller-Adler (63) has proposed a method which is based on the fact that the black substance which is formed in the bromination of histidine dissolves in ammonium carbonate solution with the formation of a blue-violet color.
- (5) Aspartic and Glutamic Acids. These amino acids are estimated by isolation as the calcium or barium salts in the presence of alcohol according to the procedure of Foreman (64) as modified by Jones and Moeller (65).
- (6) Other Amino Acids. Kapeller-Adler (66) estimates phenylalanine colorimetrically by nitrating it to 3, 4-dinitrobenzoic acid and treating the latter product with hydroxylamine in the presence of ammonia. The bluish-violet color of 3, 4-diisonitrodihydrobenzoic acid is obtained. The estimation of methionine, as given by Baernstein (67), is based on the fact that this amino acid is demethylated during the hydrolysis of the protein with hydriodic acid. The resulting homocysteine is oxidized with sodium tetrathionate. Fürth and Minnebeck (68) propose to estimate proline and hydroxyproline in proteins by a combination of the Van Slyke amino nitrogen technique, extraction with butyl alcohol, and precipitation by cadmium chloride. The color which is formed when proline and hydroxyproline are treated with an acetic acid solution of isatin is the basis for the estimation of these amino acids in the procedure which has been described by Grassmann and Arnim (69).

Several methods have been proposed for the estimation of gly-

cine. Bergmann and Fox (70) make use of the complex which is formed between glycine and potassium trioxalatocobaltiate. Patton (71) determines glycine by adding benzaldehyde to the hydrolysis mixture after the protein has dissolved. Tryptophane is thus destroyed. An adaptation of Klein and Linser's reaction (72), in which a color is produced when o-phthalic aldehyde is added, is then employed. Fürth, Scholl and Herrmann (73) make use of the conversion of alanine into lactic acid by means of nitrous acid as a means of determining this amino acid in protein hydrolysates. They estimate 0.1 to 0.5 mg. of alanine with an accuracy of ± 4 per cent.

Various investigators have summarized the results of protein analyses in tabular form. Many of these tables are summaries of the results of special analyses of certain proteins for specific amino acids. For example, there are many available tables which give the tyrosine and tryptophane content of a large number of proteins. There are other available summaries which give: the basic amino acid content of proteins; the results of the Van Slyke distribution method; the amino acids which are present in vegetable proteins; the amino acid content of animal proteins; and the amino acid content of proteins in general. The data which are given in Table IV are representative of these various summaries.

3. RETROSPECT

A point that the student of protein chemistry should bear in mind, especially when he makes use of tables which give the amino acid content of proteins, is that nearly all of the analyses which have been reported in the literature were carried out on proteins which were relatively impure. Those who have had extensive experience in the study of the chemistry of proteins are loath to state when a protein is "pure."

Granted, however, that a protein can be obtained in a pure state, that is, free from all other proteins as well as from extraneous organic and inorganic material, the question as to when the product is free from the last trace of moisture still remains. A preparation may be dried to constant weight at a certain temperature, but if the temperature should be increased by a few degrees, almost invariably more moisture will be lost. At present it is not possible to state when all of the moisture, which is not an integral part of the molecule, has been removed, and when some of the moisture which is obtained results from decomposition of the protein. This factor

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TABLE
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Insulin (Crystalline)		30.0 12.0	.6 21.0			3.0	200	88.0
Pepsin (Crystalline)		10.3 2.2	18.6	6.8		•	8.8	53.0
Histone (Thymus)	3.5	5.23	χĊ	1.5			7.00	75.3
Gluten (Wheat)	0.3	1.0	24.0	0.7			2.22	44.27
Glutenin (Wheat)	0.4 0.2 0.2	6.0 2.0 1.7	25.72	4.20 2.4	0.7			68.64
nimla2	4.3	and the second second		11.0	7.8	04	•	110 5
Bence-Jones protein	1.7 4.5 5.6	0.4.0 6.8 5.2	8.0	2.2			5.6	-
Gelatin	25.5 8.7 0.0	7.1 1.4 0.0	5.8	0.08.0.4.0.0		. 0	0.00.4	01 6 71
Hemoglobin	2.2	29.0 4.2 3.2 1.3	1.7	4.62-			8.1	10
Gliadin (Wheat)	0.0 3.0 4.	6.6 2.4 1.1	43.7	2.6 0.8 2.2			5015	07 0 71
Zein	0.0 9.8 1.9	25.0 7.6 5.9 0.2	31.3	2.5 1.8 9.0			0.00	108 8 8
Vitellin	1.1 2.2 4.2	1.3 1.3 1.3	12.2	0.5		•	0.024	1 2 2
Gasein	0.5 1.9 7.9	2.20	+ 21.8	10.5 4.1 9.0			26.20	1
Fibroin (Silk)	40.5	2.5 11.5 11.0		1.0	1.8		0.3	7 70
Keratin (Wool)	0.4.6 4.8	11.5 4.8 1.8	12.9	2.4 4.4	•	•	20.7	10 07
*(TisH) nits19X	1.5	7.1		3.4		* *	20.0	0
Globulin (Hemp) Edestin	8.00 8.00 8.00 8.00 8.00 8.00	14.5 13.1 2.5 2.5	19.2	10.2			5010101 0010100	01
Globulin (Serum)	2.2	18.7 3.8 6.7 2.3	8.2	2.2		•	000 1000	
Albumin (Wheat) Leucosin	0.9	11.3 3.8 3.3 1.2	6.7	85 83 85 63			0221- 087-4	0
Albumin (Milk) Lactalbumin	44.8	14.0 1.3 2.0 2.7	12.9	10.0 9.3 8.8		• • •	2 2 8 1 2 1 8 6 6	10
(mured) nimudla	0.0	20.0 3.1 4.7 0.5	7.7	3.1	9.0		4-1-6 8:2:0	1
(BZA) nimudlA	2.2	10.7 5.1 1.3	14.0	1.4 6.1		4.6	01.01 24.44	
	GlycineAlanine	Leucine and 180- leucine Phenylalanine Tyrosine	β-Ĥŷdrôxy-α-ami- no-n-butyric acid Glutamic acid	β-Hydroxyglu- tamic acid Aspartic acid Proline	Hydroxyproline Serine Hydroxyvaline	Cystine	Arginine Histidine Lysine	

* Human hair. 3(See also, Cohn, E. J., Brgeb. Physiol., 33, 781 (1931).)

can conceivably vary with each protein preparation under investigation. It must also be kept clearly in mind that the dried, purified product which is used in the laboratory may, at times, be quite different from that which originally occurred in the plant or the animal tissue.

It should be emphasized again that the calculation of the percentages of amino acids in proteins does not take into account the addition of water during the hydrolysis. It is for that reason that the sum of the percentages of all of the amino acids from a given protein should add to more than 100 per cent.

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CHAPTER V

THE RELATION OF THE AMINO ACIDS TO PRODUCTS OF BIOCHEMICAL IMPORTANCE

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Not only do the amino acids function as the building stones for the synthesis of proteins, but many of them play special rôles in metabolic processes such as in reactions of detoxication and as precursors of physiologically important products found both in animal and in plant life. Moreover, they serve as fuel for the animal organism since, except in periods of growth, the body does not store amino nitrogen beyond its actual needs for the synthesis of new tissue, the excess being burned and the nitrogen excreted in various forms, chiefly as urea.

1. DEAMINATION OF AMINO ACIDS (1, 2)

The breakdown of amino acids by animal tissues consists in deamination with the formation of ammonia which is subsequently converted to urea. The carbon-containing residue may be either oxidized to form carbon dioxide and water, or it may be converted to carbohydrate.

Deamination may conceivably be either hydrolytic or oxidative. Taking alanine as an example:

$$\begin{array}{ccc} \mathrm{CH_3 \cdot CH(NH_2) \cdot COOH} & \xrightarrow{H_2\mathrm{O}} \mathrm{CH_3 \cdot CH(OH) \cdot COOH} + \mathrm{NH_3} & \mathrm{or,} \\ & & \mathrm{Alanine} & \mathrm{Lactic\ acid} \\ & \mathrm{CH_3 \cdot CH(NH_2) \cdot COOH} & \xrightarrow{\mathrm{O_2}} \mathrm{CH_3 \cdot C : O \cdot COOH} + \mathrm{NH_3} \end{array}$$

Alanine Pyruvic acid

The weight of evidence points to oxidative deamination in the body with the intermediate formation of α -ketonic acids. The oxidation does not take place directly, but probably in accordance with the Wieland theory, as follows:

 $CH_3 \cdot CH(NH_2) \cdot COOH \rightarrow CH_3 \cdot C : (NH) \cdot COOH + H_2$ Alanine Imino acid

or,

$$CH_3 \cdot CH(NH_2) \cdot COOH \rightarrow CH_3 \cdot C(OH)NH_2 \cdot COOH$$
Alanine Imino acid hydrate

The imino acid hydrate splits off ammonia and is converted to the ketonic acid,

$$CH_3 \cdot C(OH)NH_2 \cdot COOH \rightarrow NH_3 + CH_3 \cdot C : O \cdot COOH$$

Imino acid hydrate Pyruvic acid

Another possibility is:

$$\begin{array}{ccc} \mathrm{CH_3 \cdot CH(NH_2) \cdot COOH} & \xrightarrow{\mathrm{O_2}} \mathrm{CH_3 \cdot CH(NH_2 \colon O_2) \cdot COOH} \\ & & \mathrm{Alanine} & & \mathrm{Amino\ acid\ peroxide} \end{array}$$

$$\begin{array}{ll} \text{CH}_3 \cdot \text{C} \colon (\text{NH}) \cdot \text{COOH} & \xrightarrow{\text{H}_2\text{O}} \text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{NH}_3 \\ \\ \text{Imino acid plus H_2O_2} & \text{Pyruvic acid} \end{array}$$

The α -ketonic acid may be converted into glucose or it may be oxidized according to the following reactions:

Deamination may be accomplished in vitro at a weakly acid reaction in the presence of p-nitrophenylhydrazine whereby glyoxal is formed. Water is then taken up in accordance with the reaction (a so-called internal Cannizzaro reaction):

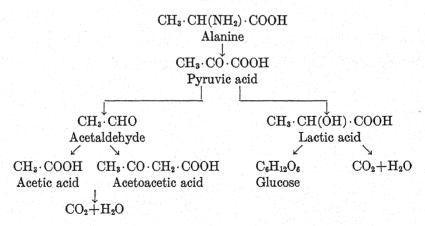
or,

A further possibility lies in the following reactions which, however, have not yet been shown to occur in the animal organism:

$$\begin{array}{c} \operatorname{CH_2(NH_2) \cdot CONH \cdot CH(CH_2OH) \cdot COOH} & -\operatorname{H_2O} \\ & \operatorname{Glycyl-serine} \\ & \operatorname{CH_2OH} \\ & \operatorname{Hydrolysis} \\ \operatorname{CO--NH--CH} & \xrightarrow{\operatorname{Hydrolysis}} \\ \operatorname{CH_2--NH--CO} \\ \operatorname{3-Methanol-2, 5-} \\ \operatorname{dioxy-piperazine} \\ \operatorname{NH_3+CH_3 \cdot CO \cdot CONH \cdot CH_2 \cdot COOH} & +\operatorname{H_2O} \\ & \operatorname{Pyruvylglycine} \\ \operatorname{CH_2(NH_2) \cdot COOH} + \operatorname{CH_3 \cdot CO \cdot COOH} \\ \operatorname{Glycine} & \operatorname{Pyruvic acid} \\ \end{array}$$

2. FORMATION OF GLUCOSE FROM AMINO ACIDS

Alanine, after deamination, can be converted into glucose in the body. This transformation, as well as the oxidation of the carbon-containing residue, may be represented as follows:



The following amino acids are considered as being capable of conversion to glucose (glycogenic) in the body: glycine, alanine, serine, cystine, aspartic acid, glutamic acid, β -hydroxyglutamic acid, proline, hydroxyproline, norleucine, and arginine.

3. FORMATION OF ACETONE FROM AMINO ACIDS

Leucine may be used to illustrate the formation of acetone from an amino acid. The reaction probably proceeds as follows: $(CH_3)_2CH \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ $Leucine \qquad \qquad (Oxidative deamination)$ $(CH_3)_2CH \cdot CH_2 \cdot CO \cdot COOH$ $\alpha\text{-Ketoisocaproic acid} \qquad (Oxidation)$ $(CH_3)_2CH \cdot CH_2 \cdot COOH$ $Isovaleric acid \qquad (\beta\text{-Oxidation})$ $(CH_3)_2 \cdot C(OH) \cdot CH_2 \cdot COOH$ $\beta\text{-Hydroxyisovaleric acid}$ $CH_3 \cdot CO \cdot CH_3$ Acetone

Leucine, isoleucine, tyrosine, and phenylalanine are considered to be subject to transformation into acetone. Tryptophane, lysine, and possibly histidine are considered to be neither glycogenic nor ketogenic.

4. INTERMEDIATE METABOLISM OF THE AMINO ACIDS

The intermediate metabolism of most of the amino acids is by no means as simple as that of alanine. Not all of the steps concerned in the breakdown of the amino acids in the body have been brought to light. The examples which are given below give some idea of the present concepts of these reactions.

l-Serine is quantitatively converted into glucose in the phloridizinized animal. Its breakdown may be represented as follows:

 $\begin{array}{ccc} {\rm CH_2OH \cdot CH(NH_2) \cdot COOH \longrightarrow CH_2OH \cdot CO \cdot CHO + NH_3 \longrightarrow} \\ {\rm Serine} & \alpha\text{-Keto-β-hydroxy-} \\ & {\rm propionic\ aldehyde} \end{array}$

Glucose (in the diabetic animal) $\begin{array}{c} \text{CH}_2\text{OH} \cdot \text{CHOH} \cdot \text{CHO} \\ \\ \text{Glyceric aldehyde} \end{array} \begin{array}{c} \text{CO}_2 + \text{H}_2\text{O} \text{ (in the normal animal)} \end{array}$

The intermediate steps in the metabolism of aspartic acid are not known. The following are possibilities:

$$\begin{array}{c} \text{HOOC} \cdot \text{CH} \colon \text{C}(\text{NH}_2) \cdot \text{COOH} \longrightarrow \\ \text{Aminofumaric acid} \\ \text{NH}_3 + \text{HOOC} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{COOH} \longrightarrow \\ \text{Ketosuccinic acid} \\ \text{HOOC} \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} \\ \text{Aspartic acid} \\ \\ \text{CO}_2 + \text{CH}_3 \cdot \text{CO} \cdot \text{COOH} \longrightarrow \text{Glucose} \\ \text{Pyruvic acid} \\ \text{HOOC} \cdot \text{CH}_2 \cdot \text{CHOH} \cdot \text{COOH} \longrightarrow \\ \\ \text{Malic acid} \\ \text{HOOC} \cdot \text{CH}_2 \cdot \text{CH}_2(\text{OH}) + \text{CO}_2 \longrightarrow \text{Glucose} \\ \\ \beta\text{-Lactic acid} \\ \end{array}$$

It appears that histidine is one of the sources of the purines since rats which have been maintained on a histidine-free diet excrete less uric acid and allantoin than those which have been fed a normal diet. β -Imidazole lactic acid, however, can replace histidine in the diet of rats. The following formulas bring out the relationship between these compounds:

A possible route in the metabolism of histidine is:

One of the facts which is known about the intermediate metabolism of tryptophane is that it can be converted into indolepyruvic acid. The latter can be substituted for tryptophane in the diet. The second is that certain animals excrete kynurenic acid in their urine when tryptophane-containing proteins are included in the diet. Kynurenic acid is undoubtedly derived from tryptophane. The following represents a possible scheme for the synthesis of kynurenic acid (3):

The conversion of tryptophane into kynurenic acid is one of the rare illustrations of the conversion of an indole into a quinoline ring.

A second compound which has been isolated from the urine of rabbits is kynurenin which appears to be an intermediate product between tryptophane and kynurenic acid:

The reactions which lead to the formation of kynurenic acid make no provision for the formation of kynurenin. It is possible that the synthesis of both compounds represents side reactions in the normal breakdown of tryptophane.

It is believed that the first step in the metabolism of phenylalanine is its conversion into tyrosine. However, a certain amount may be oxidized to phenylpyruvic acid. The metabolism of tyrosine may be represented by the following equations:

$$HO \longrightarrow CH_2 \cdot CO \cdot COOH$$

$$P-Hydroxyphenylpyruvic acid$$

$$HO \longrightarrow CH_2 \cdot CH(NH_2) \cdot COOH$$

$$OH$$

$$2:5 \text{ Dihydroxyphenylpyruvic acid}$$

$$OH$$

$$2:5 \text{ Dihydroxyphenylpyruvic acid}$$

$$HO \longrightarrow CH_2 \cdot CO \cdot COOH \longrightarrow OH$$

$$2:5 \text{ Dihydroxyphenylpyruvic acid}$$

$$HO \longrightarrow CH_2 \cdot COOH \longrightarrow OPENING of ring \longrightarrow CO_2 + H_2O$$

$$OH$$

$$Homogentisic acid$$

It is possible that the opening of the ring occurs in p-hydroxyphenylpyruvic acid yielding

The formation of keto acids from tyrosine, in its latter stages, may be represented as follows:

$$HO \longrightarrow CH_2 \cdot COOH \longrightarrow = CH \cdot COH : CH \cdot CH \stackrel{\vdots}{=} COH \cdot CO \cdot CH_2 \cdot COOH$$

$$CO_2 + H_2O \qquad : CH_3 \cdot CO \cdot CH_2 \cdot COOH$$

$$p\text{-Hydroxyphenylacetic acid} \qquad Acetoacetic acid$$

The compound, homogentisic acid, is of special interest. It occurs in the urine of alcaptonuric individuals. This is one of the con-

ditions which is known as 'an inborn error of metabolism,' and is hereditary. The urine, on standing, assumes a dark red color. Another possible reaction leading to the formation of homogentisic acid is:

The structural relationship of proline, hydroxyproline, glutamic acid, and ornithine, which is derived from arginine, suggests that these compounds share a common fate in their metabolism.

$$\begin{array}{ccc} HOOC \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH & NH_2 \cdot CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \\ & Glutamic \ acid & Ornithine \\ \end{array}$$

$$\begin{array}{ccccc} \text{CH}_2 \cdot (\text{CH}_2)_2 \cdot \text{CH} \cdot \text{NH} & \text{CH}_2 \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{NH} \\ & \text{COOH} & \text{COOH} \\ & \text{Proline} & \text{Hydroxyproline} \end{array}$$

A possible route which is followed in the breakdown of proline is:

Although the steps in the metabolism of glycine are not definitely known, the following reactions appear possible:

$$\begin{array}{c} \text{HOOC} \cdot \text{CH}_2\text{NH}_2 \rightarrow \text{HOOC} \cdot \text{CH} = \text{NH} \rightarrow \\ \text{OH} \\ \text{HOOC} \cdot \text{C} - \text{NH}_2 \rightarrow \text{NH}_3 + 2\text{CO}_2 + \text{H}_2\text{O} \\ \text{H} \end{array}$$

5. SYNTHESIS OF UREA

As has already been indicated, the metabolism of the amino acids involves the formation of ammonia. This is the normal end product of nitrogen metabolism by bacteria and protozoa (4). In mammals, amphibia, and most fishes, the end product of protein metabolism is urea; in birds and reptiles, it is uric acid.

The classical concept of the formation of urea is represented by

$$\begin{array}{c} NH_4O \\ NH_3+CO_2+H_2O \rightarrow \\ NH_4O \\ \hline \\ NH_4O \\ \hline \\ Ammonium \\ carbonate \\ \hline \\ NH_4O \\ \hline \\ NH_2 \\ \hline \\ NH_2 \\ \hline \\ Ammonium \\ carbamate \\ \hline \\ Urea \\ \hline \\ Urea \\ \hline \end{array}$$

Through the brilliant efforts of Krebs and his associates (5), it is now believed that arginine plays the chief rôle in urea synthesis. According to this concept the chemical reactions are as follows:

$$\begin{array}{c} \mathrm{NH_2 \cdot CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH} \xrightarrow{\phantom{Cooh$$

$$\text{NH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}_2 \cdot (\text{CH}_2)_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} \xrightarrow{\text{NH}_3}$$
Citrulline

$$\begin{array}{c} \mathrm{NH_2 \cdot C: (NH) \cdot NH \cdot CH_2 \cdot (CH_2)_2 \cdot CH (NH_2) \cdot COOH} \xrightarrow{\mathrm{Arginase}} \mathrm{urea} + \mathrm{ornithine} \\ \mathrm{Arginine} \end{array}$$

From ornithine the cycle may start anew. The conversion of arginine to ornithine is brought about by the action of arginase, an enzyme which occurs in the liver. Bollman, Mann and Magath (6) have shown that synthesis of urea in the dog ceases when the liver is removed, indicating that the locus of urea synthesis is in this organ.

The rate of the synthesis of urea, when the tissue slice technique is used, increases rapidly with increasing concentrations of the bicarbonate-CO₂-buffer of the medium. This is explained on the basis of the reactions:

$$\begin{array}{c} \mathrm{NH_2CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH} \\ \\ \mathrm{Ornithine} \\ & \downarrow + \mathrm{CO_2} \\ \\ \mathrm{HOOC \cdot NH \cdot CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH} \\ \\ \delta\text{-Carbamino ornithine} \\ & \downarrow + \mathrm{NH_3} \\ \\ \mathrm{NH_2 \cdot CO \cdot NH \cdot CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH} \\ \\ \mathrm{Citrulline} \end{array}$$

(Acid amide of the carbamino acid of ornithine)

Evidence for the synthesis of amino acids, illustrated by their indispensability in feeding experiments, is given in Chapter XVIII. Reactions illustrating the synthesis of amino acids in plants and animals are given in Chapter II.

6. RELATIONSHIP OF AMINO ACIDS TO OTHER COMPOUNDS FOUND IN THE BODY

The close chemical relationship between arginine and creatine and creatine suggests that this amino acid may be the precursor of these two substances. Creatine is found in muscle as creatine phosphoric acid, and creatinine is a normal constituent of urine. In the pre-adolescent period, creatine is excreted in the urine. The synthesis of creatine and creatinine is supposed to follow the reactions:

$$\begin{array}{c} \mathrm{NH_2 \cdot C: (NH) \cdot NH \cdot CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH} \xrightarrow{O_2} \\ \\ \mathrm{Arginine} \end{array}$$

$$NH_2 \cdot C : (NH) \cdot NHCH_2 \cdot COOH \xrightarrow{\text{methylation}}$$
Guanidine acetic acid

$$\begin{array}{c} \text{NH}_2 \cdot \text{C} \colon (\text{NH}) \cdot \text{N}(\text{CH}_3) \cdot \text{CH}_2 \cdot \text{COOH} \xrightarrow{\qquad \qquad -\text{H}_2\text{O}} \\ \\ \text{Creatine} \\ \text{NH} \xrightarrow{\qquad \qquad \text{CO}} \end{array}$$

$$HN = C$$
 $N(CH_3) - CH_2$
 $Creatinine$

The views regarding the precursor of creatine and creatinine are, however, so conflicting that the above must be regarded as a speculative hypothesis rather than a proven fact. A full account of this subject is given by Hunter (7).

A more recent view of the origin of creatinine is the following:

$$\begin{array}{c} \mathrm{CH_2 \cdot (NH_2) \cdot COOH} + \mathrm{O} : \mathrm{C(NH_2)_2} \rightarrow \\ \mathrm{(Glycine)} & \mathrm{(Urea)} \end{array}$$

 $NH_2 \cdot C : (NH) \cdot NHCH_2 \cdot COOH \xrightarrow{\text{methylation}}$ (Guanidine acetic acid)

 $NH_2 \cdot C : (NH) \cdot N(CH_3) \cdot CH_2 \cdot COOH.$ (Creatine)

Meyerhof and Lohmann (8) have reported that, instead of creatine phosphoric acid, invertebrate muscle contains arginine phosphoric acid. The formulas of these compounds are:

$$\begin{array}{ccc} NH \cdot PO(OH)_2 & NH \cdot PO(OH)_2 \\ HN : C & HN : C \\ & N(CH_3) \cdot CH_2 \cdot COOH & NH \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH \\ Creatine phosphoric acid & Arginine phosphoric acid \end{array}$$

Evidence that these substances participate in muscular activity is deduced from the fact that, on stimulation of muscle, inorganic phosphoric acid increases and creatine phosphoric acid decreases. Both creatine phosphoric acid and arginine phosphoric acid appear to undergo decomposition during the excitation stage rather than during contraction. In invertebrates, arginine phosphoric acid appears to play the rôle which is taken by creatine phosphoric acid in vertebrates.

A number of other substances occur in urine which owe their origin to amino acid precursors. By bacterial action, indole and skatole are produced from tryptophane. These products appear in part in the feces and in part are excreted in the urine conjugated with sulfuric acid as indoxyl or skatoxyl sulfuric acid. The derivation of these products from tryptophane is shown in the following reactions:

The amino acid, cystine, deserves special consideration. In the first place, it is probably the precursor of taurine which occurs conjugated with cholic acid as one of the bile acids. Friedmann (9) has suggested that the conversion of cystine to taurine takes place in accordance with the reactions indicated by the following equations:

$$\begin{array}{c|cccc} CH_2-S-S-CH_2 & CH_2SH & CH_2SO_3H \\ \hline CH(NH_2) & CH(NH_2) & CH(NH_2) & CH(NH_2) \\ \hline COOH & COOH & COOH & COOH \\ \hline Cystine & Cysteine & Cysteic acid \\ \hline NH_2\cdot CH_2\cdot CH_2\cdot SO_2OH + & Taurocholic acid \\ \hline Taurine & Taurocholic acid \\ \hline \end{array}$$

The work of Gortner and Hoffman (10), Lewis and Lewis (11), as well as unpublished work which has been carried out by Miss Morey in this laboratory, indicates that the yields of taurine obtained by Friedmann's procedure are extremely poor.¹ Another possibility is that cystine is converted to thiolethylamine, $HS \cdot CH_2 \cdot CH_2 \cdot NH_2$, and this, in turn, to taurine. Glycine is likewise conjugated with cholic acid to yield glycocholic acid, another bile acid.

Butz and du Vigneaud (12) have been able to convert methionine into homocystine, the higher homologue of cystine, by heating it in sulfuric acid. The reactions are:

$$\begin{array}{c} \mathrm{CH_3 \cdot S \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \longrightarrow HS \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH \longrightarrow} \\ \mathrm{Methionine} & \mathrm{Homocysteine} \end{array}$$

$$\begin{array}{c} \text{HOOC} \cdot \text{CH}(\text{NH}_2) \cdot \text{CH}_2 \cdot \text{CH}_2 \text{--S} \text{--S} \text{--CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} \\ \text{Homocystine} \end{array}$$

Homocystine promotes growth in rats when added to a cystine-deficient diet.

The discovery of glutathione in plant and animal tissues by Hopkins (13) emphasized the importance of cystine in connection with the subject of physiological oxidation. Solutions of cysteine when made slightly alkaline, on exposure to air, are readily oxidized to cystine. The reverse process occurs almost as readily in the presence of mild reducing agents. Accordingly, cysteine and cystine constitute an auto-oxidizable system, the cysteine being oxidizable by molecular oxygen, and cystine being reducible by hydrogen.

$$\begin{array}{c|cccc} CH_2 \cdot S - S - CH_2 & CH_2SH \\ & & H_2 & \\ CH(NH_2) & CH(NH_2) & \rightleftarrows & CH(NH_2) \\ & & O_2 & \\ COOH & COOH & COOH \\ & Cystine & Cysteine \\ \end{array}$$

The constitution of glutathione has been established by Hopkins (14) and by Kendall and his associates (15) as a tripeptid of glutamic acid, cysteine, and glycine, having the chemical formula:

¹ White, A., and Fishman, J. B., J. Biol. Chem., 116, 457 (1936) report that cysteic acid is converted to taurine at 235-240°.

 $\begin{array}{c} {\rm COOH \cdot CH(NH_2) \cdot CH_2 \cdot CH_2 \cdot CONH \cdot CH(CH_2 \cdot SH) \cdot CONH \cdot CH_2 \cdot COOH} \\ {\rm Glutaminyl-cysteyl-glycine~(reduced~form~of~glutathione)} \end{array}$

The changes which glutathione undergoes in oxidation may be represented as follows (16):

$$\begin{array}{c} \text{G-SH+HS-G} \\ \downarrow + \text{O}_2 \\ \text{GS-----SG+H}_2 \text{O}_2 \\ \downarrow + \text{H}_2 \\ \text{G-SH+HS-G} \end{array}$$

In certain pathological conditions, cystine is found in the kidney or in the bladder in the form of cystine stones. Normally the sulfur in cystine is oxidized to sulfates which are eliminated by the kidney. In certain individuals, cystine appears in the urine. This condition, which has been termed cystinuria, appears to be hereditary and has been characterized by Garrod (17) as an 'Inborn Error of Metabolism.'

The demonstration by Abel (18) and his co-workers that insulin, the hormone of the islet cells of the pancreas, contains cystine may be of possible significance with respect to its mode of action. According to recent analysis (19), crystalline insulin contains 12 per cent of tyrosine, 12 per cent cystine, 21 per cent glutamic acid, 30 per cent leucine, 3 per cent arginine, 2 per cent lysine, and 8 per cent histidine. Tryptophane appears to be absent.

7. THE RÔLE OF AMINO ACIDS IN DETOXICATION

The amino acids play special rôles in the reactions of detoxication of certain poisonous substances when they are introduced into the animal body. A review of this subject has been presented by Ambrose and Sherwin (20). On feeding benzoic acid to a dog or to man, it is in part conjugated with glycine and appears in the urine as hippuric acid.

$$\begin{array}{ccc} \text{C}_6\text{H}_5 \cdot \text{COOH} + (\text{NH}_2)\text{CH}_2 \cdot \text{COOH} \rightarrow \text{C}_6\text{H}_5 \cdot \text{CONH} \cdot \text{CH}_2 \cdot \text{COOH} \\ \text{Benzoic acid} & \text{Glycine} & \text{Hippuric acid} \end{array}$$

Another method of detoxicating benzoic acid consists in conjugation with glucuronic acid with the formation of benzoyl glucuronic acid.

Phenylacetic acid is detoxicated by conjugation with glycine and is excreted by the dog as phenaceturic acid. It appears that the amount of conjugation which has been found to take place when benzoic acid is fed exceeds the amount of glycine which may be available in the body at any particular time and this has been regarded as evidence that glycine may be synthesized in the body. When benzoic acid is fed to fowls it is conjugated with ornithine to form ornithuric acid which appears in the excreta.

$$\begin{array}{ll} 2\mathrm{C}_6\mathrm{H}_5 \cdot \mathrm{COOH} + \mathrm{NH}_2\mathrm{CH}_2 \cdot (\mathrm{CH}_2)_2 \cdot \mathrm{CH}(\mathrm{NH}_2) \cdot \mathrm{COOH} {\longrightarrow} \\ \mathrm{Benzoic\ acid} & \mathrm{Ornithine} \end{array}$$

 $C_6H_5 \cdot CONH \cdot (CH_2)_3 \cdot CH(COOH) \cdot CONH \cdot C_6H_5$ Di-benzoyl ornithine (ornithuric acid)

Phenylacetic acid, p-nitrophenylacetic acid, and pyromucic acid are similarly detoxicated by birds. Curiously, while the dog conjugates phenylacetic acid with glycine to form phenaceturic acid, in man it is combined with glutamine, the amide of glutamic acid, and is excreted as phenylacetylglutamine:

$$\begin{array}{ll} \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{COOH} + \text{COOH} \cdot \text{CH(NH}_2) \cdot (\text{CH}_2)_2 \cdot \text{CONH}_2 \\ \text{Phenylacetic acid} & \text{Glutamine} \end{array}$$

 $\begin{array}{c} \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CONH} \cdot \text{CH}(\text{COOH}) \cdot (\text{CH}_2)_2 \cdot \text{CONH}_2 \\ \text{Phenylacetylglutamine} \end{array}$

When bromobenzene, chlorobenzene, or iodobenzene is fed to dogs, it appears in the urine as a mercapturic acid.² The detoxicating amino acid in this case is cysteine. The probable reactions are the following:

² See Stekol, J. A., J. Biol. Chem., 117, 147 (1937); 121, 93 (1937); 122, 333 (1938) for the effects of diets of varying sulfur content upon this synthesis.

8. AMINES DERIVED FROM AMINO ACIDS (21)

While deamination constitutes the chief method whereby breakdown of the amino acid takes place, it is by no means the only one. In the higher organisms it is the only proven method. However, if we accept the conversion of cysteic acid to taurine, according to the reaction as postulated by Friedmann, we have an illustration of the partial breakdown, by splitting off carbon dioxide, of a compound whose chemical makeup closely resembles that of an amino acid. Taurine is then the amine of cysteic acid. The amino group is in the β -position. The union of taurine with cholic acid may also be regarded as a detoxication reaction.

A group of lower organisms comprising the bacteria and fungi are able to produce from amino acids a series of nitrogenous bases which arise by decarboxylation of the amino acid molecule in accordance with the general equation,

$$\begin{array}{ccc} R \cdot CH(NH_2) \cdot COOH & \xrightarrow{\quad -CO_2} R \cdot CH_2 \cdot NH_2 + CO_2 \\ & \text{Amino acid} & \text{Amine} \end{array}$$

Decarboxylation, especially under anerobic conditions, may be accompanied by reduction, in which case formic acid is produced instead of carbon dioxide:

$$\begin{array}{ccc} R \cdot CH(NH_2) \cdot COOH & \xrightarrow{\quad H_2 \quad} R \cdot CH_2 \cdot NH_2 + H \cdot COOH \\ & \text{Amino acid} & \text{Amine} \end{array}$$

At the same time that decarboxylation is taking place, deamination is also proceeding, as evidenced by the production of ammonia. The conditions which determine the relative proportions of these two processes are very complex and have not yet been fully elucidated. It has, however, been observed that the presence of carbo-

hydrates in the culture media greatly diminishes the production of ammonia, presumably because, in the absence of carbohydrates, the organisms utilize the amino acids as a source of energy as well as a source for nitrogen. In utilizing the carbon and hydrogen components, they incidentally split off ammonia as a by-product. Since it is usually quite difficult or impossible to grow an organism in a medium containing a single amino acid as the sole source for nitrogen, it has been found advantageous to include peptone or other more complex protein material. It is also necessary to prevent increase of acidity by the addition of substances such as calcium or magnesium carbonate.

In studying the production of histamine from histidine, Koessler and Hanke (22) have shown that the formation of this amine is always coincident with the production of a medium which is distinctly acid, hence it may be supposed that the amine is formed to neutralize the excess acid. The inclusion of carbohydrate favors the production of acid and thus perhaps serves to favor also the formation of the amine. They found that the production of histamine is promoted by leucine, alanine, arginine, glycine, or peptone. Glutamic acid and tryptophane are without effect, while cystine markedly reduces the yield of histamine. Of 29 strains of Bacillus coli communis investigated by Koessler and Hanke, only 6 were able to convert histidine to histamine.

When a combination of the two processes of decarboxylation and deamination proceed simultaneously, methane may be formed from glycine and n-butyric acid from glutamic acid. From diamino acids, dibasic monoamino acids, and cyclic amino acids, there may be formed by bacterial action compounds which have been termed ω -amino acids. The following are examples which illustrate the formation of these products:

(a) Partial deamination of a diamino acid, as in the formation of δ -amino valeric acid from ornithine:

 $\mathrm{NH_2} \cdot (\mathrm{CH_2})_3 \cdot \mathrm{CH}(\mathrm{NH_2}) \cdot \mathrm{COOH} + \mathrm{H_2} {\longrightarrow} \mathrm{NH_2} \cdot (\mathrm{CH_2})_4 \cdot \mathrm{COOH} + \mathrm{NH_3}$

(b) By partial decarboxylation of a dibasic amino acid, as in the production of γ -aminobutyric acid from glutamic acid:

 $COOH \cdot CH(NH_2) \cdot (CH_2)_2 \cdot COOH \rightarrow NH_2 \cdot (CH_2)_3 \cdot COOH + CO_2$

(c) By reduction of a cyclic amino acid, as in the production of δ -aminovaleric acid from proline:

$$\begin{array}{c|c} H_2C & CH_2 \\ H_2C & CH \cdot COOH + H_2 \rightarrow NH_2 \cdot (CH_2)_4 \cdot COOH \\ \hline N \\ H \end{array}$$

When a protein is broken down to the stage of amino acids, these substances are available for further synthesis of protein products. If, however, the degradation proceeds further, such as deamination or decarboxylation of the amino acid, or, if it is methylated, it is no longer available for protein synthesis, at least proteins containing such products have not yet been found. Ackermann and Kutscher (23) have given the name aporrhegma to the degradation products of amino acids. Under this term they include both the basic and the acidic products of bacterial putrefaction of protein. A partial list of the amino acids and their corresponding aporrhegmas are given in Table I.

rable 1.			
	TABLE I		
Amino Acid	Aporrhegma		
Arginine	Ornithine		
	Tetramethylenediamine		
	Agmatine		
	δ-Aminovaleric acid		
	Citrulline		
Aspartic acid	β -Alanine		
	Succinic acid		
Glutamic acid	γ-Aminobutyric acid		
Glycine	Methylamine (?)		
Histidine	β -Imidazolylethylamine		
	β -Imidazolylpropionic acid		
Leucine	Isoamylamine		
	Isovaleric acid		
Lysine	Cadaverine (Pentamethylenediamine)		
Phenylalanine	β -Phenylethylamine		
	Phenylacetic acid		
	β-Phenylpropionic acid		
Proline	Pyrrolidine		
Tryptophane	Indole		
	Skatole		
	β -Indolpropionic acid		
	Indolaceticacid		
Tyrosine	Tyramine (p-Hydroxyphenylethylamine)		
	p-Hydroxyphenylacetic acid		
	$p ext{-Hydroxyphenyl-}eta ext{-propionic acid}$		
	5는 기계하는 영화를 다고 있는 아이들은 살이 하면 바다를 하는 수 있다면 하면 하면 하면 하는 것이 되었다. 그런 하는 것은 것이 없는 것은 사람들이 없는 것이다.		

The importance of decarboxylation from a biochemical and physiological point of view arises out of the intense physiological activity of many of these products which are so formed, and the resemblance of some of these products to certain of the active principles of the glands of internal secretion.

The amines which are given in Table II have been prepared by the action of putrefactive microorganisms on the corresponding amino acids. In the case of methylamine it is not certain as to whether the precursor is glycine or choline (trimethyl-hydroxy-

ethyl-ammonium hydroxide), a constituent of lecithin. TABLE II Amino Acid Amine 1. CH₃·CH(NH₂)COOH CH₃ CH₂ NH₂ (Alanine) (Ethylamine) 2. NH₂·C:(HN)·NH·CH₂ $NH_2 \cdot C : (HN) \cdot NH \cdot CH_2(CH_2)_2 \cdot CH_2 \cdot NH_2$ $\cdot (CH_2)_2CH(NH_2)COOH$ (Arginine) (Agmatine: guanido-butylamine) 3. CH₂(NH₂)·COOH CH₃· NH₂ (Glycine) (Methylamine) 4. $_{\rm CH}$ ŇΗ ŇΗ $C \cdot CH_2 \cdot CH_2 \cdot NH_2$ HC= $C \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ (Histidine) (Histamine: β -Imidazole-ethylamine) 5. $CH_3 \cdot (C_2H_5) \cdot CH \cdot CH(NH_2) \cdot COOH \quad CH_3 \cdot (C_2H_5) \cdot CH \cdot CH_2 \cdot NH_2$ (Isoleucine) (2-Methyl-butylamine) 6. (CH₃)₂CH·CH₂·CH(NH₂)COOH $(CH_3)_2CH \cdot CH_2 \cdot CH_2 \cdot NH_2$ (Leucine) (Isoamylamine) 7. $NH_2 \cdot CH_2 \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$ NH2·CH2(CH2)3·CH2·NH2 (Lysine) (Cadaverine: Pentamethylene diamine) 8. $NH_2 \cdot CH_2 \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$ $NH_2 \cdot CH_2 \cdot (CH_2)_2 \cdot CH_2 \cdot NH_2$ (Ornithine) (Putrescine: tetramethylene diamine) 9 C₆H₅· CH₂· CH(NH₂)· COOH $C_6H_5 \cdot CH_2 \cdot CH_2 \cdot NH_2$ (Phenylalanine) $(\beta$ -Phenylethylamine) 10. $C \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ $C \cdot CH_2 \cdot CH_2 \cdot NH_2$ CHH₄Ć H₄C (Tryptophane) $(\beta$ -Indolethylamine) 11. $HO \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH$ HO · C₆H₄ · CH₂ · CH₂ · NH₂ (Tyramine: p-Hydroxyphenyl- β -ethylamine)

 $(CH_3)_2 \cdot CH \cdot CH_2 \cdot NH_2$

(Isobutylamine)

(Tyrosine)

(Valine)

12. $(CH_3)_2 \cdot CH \cdot CH(NH_2) \cdot COOH$

The aliphatic monoamines (methylamine, ethylamine, isobutylamine, isoamylamine, dimethylaminobutane) exert a physiological action simulating the effects of stimulation of the sympathetic nervous system. They have been termed by Barger and Dale "Sympathomimetic" bases. Isobutylamine is the first member of the series which, on intravenous injection, produces a distinct rise in blood pressure. The physiological activity increases with increasing length of the aliphatic hydrocarbon chain up to hexylamine and thereafter declines with increasing number of carbon atoms in the chain. The introduction of a ring structure markedly increases the physiological activity. Thus β -phenylethylamine is at least five times as active as any aliphatic amine. Two milligrams of this substance will increase the blood pressure of a cat from 130 mm. to 180 mm. The most active amine is tyramine which exerts an effect on blood pressure about one-twentieth as great as adrenaline. Tyramine causes the non-pregnant uterus to relax, while the pregnant uterus is stimulated to contraction. β-Indoleethylamine is less potent than tyramine and also differs somewhat in its action. It has a direct stimulating action on smooth muscle fibres as well as a transient stimulation of the central nervous system which leads to muscular tremors or even convulsions.

Putrescine and cadaverine possess very little physiological activity. In contrast to the monoamines, they cause a fall instead of a rise in blood pressure when injected intravenously. They have been found to occur in the urines of cystinurics, indicating perhaps defective power of the tissues to deaminize these amino acids. Agmatine has a direct action upon the uterine muscles, inducing contractions. Its effect, however, is decidedly less than that of histamine (ergamine). The latter amine occurs along with tyramine and ergotoxine in ergot preparations. Ergot is a parasitic fungus which grows on rye and has been employed since ancient times to cause contractions of the uterus. Histamine stimulates nonstriated muscle directly, inducing powerful contractions of the uterus, and also stimulates smooth muscle fibres in other organs such as the stomach, the intestines, and the constrictor muscles of the pupils of the eyes. It induces spasmodic contractions of the bronchioles when administered in relatively large doses. It may play a rôle in inducing contractions of the small intestines (24).

The ω -amino acids are relatively less important than the amines. An important representative of this group is carnosine which, next to creatine, is the most abundant nitrogenous base in meat extract.

Carnosine, on hydrolysis, yields histidine and β -alanine. Its constitution is probably represented by the formula

$$\begin{array}{c} \text{CH} \cdot \text{N} \\ \text{CH} \\ \text{H}_2\text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{NH} \cdot \text{CH}(\text{COOH}) \cdot \text{CH}_2 \cdot \text{C} \\ \text{Carnosine} \end{array}$$

β-Alanine, $NH_2 \cdot CH_2 \cdot CH_2 \cdot COOH$, is formed from aspartic acid by putrefaction. Similar treatment of glutamic acid yields γ -amino-n-butyric acid, $NH_2 \cdot (CH_2)_3 \cdot COOH$. δ-Amino-n-valeric acid, $NH_2(CH_2)_4 \cdot COOH$, is derived in putrefaction of either arginine, ornithine, or proline, while ε-amino-n-caproic acid, $NH_2 \cdot (CH_2)_5 \cdot COOH$, is similarly obtained from lysine. β-Imidazolylpropionic

$$\begin{array}{c} \mathrm{CH} = \mathrm{C} \cdot \mathrm{CH}_2 \cdot \mathrm{CH}_2 \cdot \mathrm{COOH}, \\ \downarrow & \downarrow \\ \mathrm{acid}, & \stackrel{\mathrm{N}}{N} \\ \mathrm{is} \ \mathrm{derived} \ \mathrm{from} \ \mathrm{histidine} \ \mathrm{by} \ \mathrm{putrefaction}. \end{array}$$

9. THE BETAINES (21)

Both animals and plants possess the ability to methylate various types of compounds. It is therefore not surprising to find that both amino acids and their decomposition products (aporrhegmas) occur in the form of methylated derivatives. Examples of methylated compounds which occur in the animal body are creatine and creatinine. Another is that of methyl-hydroxy-pyridine. This compound is found in the urine of a dog to which pyridine has been fed (25).

Ackermann (26) has similarly found that trigonellin occurs in the

urine of dogs after feeding nicotinic acid. The synthesis probably proceeds as follows:

In the plants, the formation of methylamino compounds is very common. The manner in which methylation is accomplished both in animals and plants is still unknown. In the case of plants it has been suggested that methylation is accomplished by means of formaldehyde, but definite proof to that effect is lacking. It has been further suggested that methylation is a process of detoxication both for the plant and the animal. The betaines are amino acids in which the nitrogen atom is united to methyl groups in place of hydrogen atoms. These substances in the absence of water form cyclic anhydrides which open when they are dissolved in water or when they unite with acids.

The α -betaines differ greatly in the ease with which trimethylamine can be split off. Some are so unstable that they cannot be prepared by the ordinary processes of methylation. Thus aspartic acid, when treated with methyl iodide and alkali, breaks up into trimethylamine and fumaric acid. Tyrosine behaves similarly. In fact, betaines of tyrosine and phenylalanine have not been found to occur in nature. The betaine of tryptophane is somewhat more stable, and ergothioneine requires heating with concentrated alkali to decompose it into trimethylamine and the unsaturated acid.

It is still an open question as to whether the betaines are waste products in the plant or whether they are necessary for the functional activities of the plant. It is significant that betaines occur most abundantly in those parts of the plant where the vegetative processes are most active.

The simplest betaine which occurs in nature is trimethyl glycine. It is found in the sap of the sugar beet, *Beta vulgaris*. It is extracted, together with the sugar, and remains in the molasses when the

sugar is refined. It is non-toxic, but, due to its apparent non-utilization, it cannot be considered a food. Betaine, when dried at a temperature above 100°, possesses the formula:

$$(CH_3)_3$$
: N CO

When it is dissolved in water it is probably represented by the formula:

$$(CH_3)_3 \stackrel{+}{:} \stackrel{C}{N} \cdot CH_2 \cdot CO\overline{O}$$

The monomethyl derivative of glycine, sarcosine, is formed when creatine is heated with alkali for a long period. Creatine may be considered as the ureide of sarcosine which, therefore, relates it to the betaines:

Stachydrine is the betaine of proline:

It was first discovered in the edible tubers of Stachys tuberifera. Stachydrine is inert physiologically; when taken by mouth it is excreted unchanged. Trimethylhistidine or hercynine was first isolated from mushroom extract. Its structure is:

Closely related to the betaine of histidine is ergothioneine, the betaine of thiolhistidine, a compound which Tanret (27) isolated from ergot. Its composition was established by Barger and Ewins (28).

$$\begin{array}{c|cccc} CH-N & CH-N \\ & & CSH \\ \hline C-NH & C-NH \\ \hline CH_2 & CH_2 \\ \hline CH(NH_2) & HC-N(CH_3)_3 \\ \hline COOH & CO-O \\ Thiolhistidine & Ergothioneine \\ \end{array}$$

The occurrence of ergothionine in animal blood raises the question as to the occurrence of thiolhistidine in proteins. Moreover, both thiolhistidine and ergothionine, due to the presence in the molecule of the sulfhydryl group, can play a rôle in an oxidizing-reducing system in the same way as cystine-cysteine or the reduced and oxidized form of glutathione.

A compound which is also closely related to histidine is anserine, a compound of methylated histidine and β -alanine. It was isolated from goose muscle by Ackermann, Timpe and Poller (29). Its constitution has been established by Linneweh, Keil and Hoppe-Seyler (30) as:

$$\begin{array}{c|cccc} HC & & CH_2 \cdot CH \cdot COOH \\ \hline & & & & \\ N & NCH_3 & NH \\ \hline & & & \\ C & & O:C \cdot CH_2 \cdot CH_2 \cdot NH_2 \\ \hline & & & \\ H & & & \\ \end{array}$$

It is interesting to note that methyl histidine is closely related to creatinine:

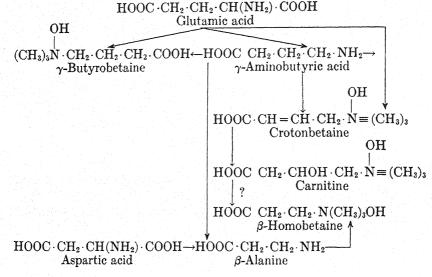
The betaine of tryptophane (hypaphorine) occurs in the seeds of *Erythrina hypaphorus*, a shade tree which grows in Java. The constitution of hypaphorine is:

Ornithine betaine (myokynin) has been isolated from the muscles of the dog, the horse, and man. Due to the presence in ornithine of two amino groups, the betaine contains therefore six methyl groups.

$$\begin{array}{c} (CH_3)_3 \\ NH_2 \cdot CH_2(CH_2)_2 \cdot CH(NH_2) \cdot COOH \\ Ornithine \\ \end{array} \begin{array}{c} (CH_3)_3 \\ N-CH_2 \cdot (CH_2)_2 \cdot CH \cdot CO \cdot O \\ HO \\ \end{array}$$

In putrefying meat a betaine has been isolated whose composition is that of γ -n-butyro-betaine, the betaine of γ -aminobutyric acid. It has an action upon nerve-endings resembling that of curare, and, when injected, produces convulsions, dyspnea, and paralysis. It also occurs in the muscles of fish and reptiles. On the other hand, carnitine and crotonbetaine, which occur in meat extracts, are almost devoid of immediate physiological activity.

The relationship between glutamic acid, the above mentioned two betaines, and certain other compounds is represented in the following diagram:



The occurrence and distribution of the betaines in the animal kingdom are shown in Table III.

TABLE III

Vertebrates:		aine Other Betaines and Nitrogenous Bases
Mammals Reptiles Amphibians		Carnitine, γ -butyrobetaine, N-methyl pyridine γ -Butyrobetaine
Teleosts	+	γ-Butyrobetaine, trimethylamine oxide in salt water fish
Chondrostei	+	
Selachians	+	
Cyclostomes	+	
Invertebrates:		
Arthropods:		
Insects		
Crustacea	+	Trimethylamine oxide, N-methyl pyridine
Molluscs:		
Pelecypods (mussels)	+	γ-Butyrobetaine, stachydrine, N-methyl pyridine
Cephalopods	+	Carnitine, trimethylamine oxide
Annelids	+	
Platyhelminths	+	
Echinoderms:		
Holothuroids	+	
Echinoids		Trigonelline
Coelenterates:		맛있다. 그리는 그리고 아니는 사람이 나를 보고 있다.
Hydrozos		Trigonelline (?)
Anthozos		γ-Butyrobetaine, N-methyl pyridine
Porifers	+	
(Kutscher, F., and Ack	ermanı	n, D., Annual Rev. Biochem., 2, 358, (1933).)

10. ADRENALINE AND EPHEDRINE

Although the origin of adrenaline is not known, the close relationship of this substance to tyrosine and dihydroxyphenylalanine suggests that these amino acids may be the precursors of the blood pressure raising principle of the adrenal glands. It has been suggested that the synthesis of adrenaline may follow the course of the following reactions:

$$HO \longrightarrow CH_2 \cdot CH(NH_2) \cdot COOH \text{ or } HO \longrightarrow CH_2 \cdot CH(NH_2) \cdot COOH \longrightarrow Tyrosine \qquad 3, 4-Dihydroxyphenylalanine \\ HO \longrightarrow CH(OH) \cdot CH(NH_2) \cdot COOH \longrightarrow 3, 4-Dihydroxyphenyl-serine \\ HO \longrightarrow CH(OH) \cdot CH(NHCH_3) \cdot COOH \longrightarrow 3, 4-Dihydroxyphenyl-methylamino-serine \\ HO \longrightarrow CH(OH) \cdot CH_2 \cdot NH \cdot CH_3$$

Adrenaline has been synthesized according to a number of methods. One of these consists in condensing catechol with monochloracetic acid. The resulting chloracetocatechol is treated with aqueous methylamine solution whereby methylaminoacetocatechol is formed. This is subsequently reduced to adrenaline.

Adrenaline

When injected intravenously, adrenaline causes a marked rise in blood pressure. Local injections constrict the adjacent blood vessels so that absorption is delayed and its action is prolonged. The extensive use of adrenaline in minor surgery to prevent bleeding is based upon this property. Injection of adrenaline leads to glucohemia and glycosuria. It is believed that violent emotions such as fear and anger lead to an increase in the discharge of adrenaline with a resulting glycosuria. Like tyrosine and dihydroxyphenylalanine, adrenaline is a reducing agent. It is readily converted by oxidizing agents and oxidizing ferments into deeply colored pigments. It is possible that the bronze-like pigmentation of the skin in Addison's disease is due to an adrenaline derivative. Various other pigments such as that which occurs in the ink-sac of the cephalopod Sepia and in ochronosis may have as precursors the hydroxyphenyl amino acids or their derivatives.

The precursor of ephedrine and of pseudoephedrine, substances which occur in *Ephedra vulgaris*, is probably phenylalanine, as can be seen from the close structural relationship.

The physiological action of ephedrine resembles that of adrenaline in some respects. As a vasoconstrictor its action is less intense, although more prolonged than that of adrenaline. It possesses a characteristic mydriatic action.

11. THYROXINE

The physiological action of the amino acid thyroxine is so characteristic and so pronounced, and its rôle in life processes is so important as to necessitate some comment. Thyroxine is found combined with other amino acids as thyroglobulin in the thyroid gland. The fact that diiodotyrosine has also been found in the thyroid gland (31) suggests that thyroxine may be formed from this amino acid. This point, however, has not been proven. Moreover, the fact that administration of inorganic iodides prevents certain types of goiter points to the synthesis of diiodotyrosine in the body from tyrosine or less probably from phenylalanine.

Hypothyroidism is expressed by the condition of myxedema. There is a marked reduction in the basal metabolic rate and a tendency to obesity and mental sluggishness. If the hypofunction occurs during fetal life or childhood, the condition of cretinism results. Hyperfunction of the thyroid gland is characterized by an increased basal metabolic rate, increased temperature and heart rate, nervousness, and hyperexcitability.

12. RELATIONSHIP OF CERTAIN AMINO ACIDS TO THE PURINES

The close structural relationship of histidine to the purine compounds, together with the fact that the Dalmatian coach hound excretes uric acid when maintained on a purine free diet, suggests the possibility of histidine acting as a precursor for purine synthesis.

Rose and Cook (32) have shown that there is a marked reduction in the output of allantoin by rats when deprived of histidine in the diet. When the diet was supplemented by the addition of histidine, an increased excretion of allantoin followed.

Uric Acid

A discussion of the rôle played by the amino acids in nutrition is given in Chapter XIX.

13. DISSOCIATION CONSTANTS OF THE AMINES AND THE BETAINES

The dissociation constants of some of the compounds which have been mentioned in this chapter are given in Table IV.

TABLE IV

Apparent Dissociation Constants of Amines, Betaines, and Certain Other Compounds at 25°

	$K_a{}'$	K_b'
(a) Amines		
Ammonium hydroxide		1.8 ×10 ⁻⁵
Diethylamine		1.26×10^{-3}
Dimethylamine		7.4×10^{-4}
Ethylamine		5.6×10^{-4}
Isoamylamine		5×10^{-4}
Isobutylamine		3.1×10^{-4}
Methylamine		5×10^{-4}
Triethylamine		6.4×10^{-4}
Trimethylamine		7.4×10^{-5}
(b) Betaines		
Dimethylglycine	1.4×10^{-10}	1.1×10^{-12}
Glycine betaine	1 to 1.33×10^{-14}	7.6 to 8.7×10^{-13}
Sarcosine	$0.98 \text{ to } 1.3 \times 10^{-10}$	1.7 to 1.8 \times 10 ⁻¹²
Sarcosyl-glycine	3.1×10^{-9}	1.3 ×10 ⁻¹¹
Sarcosyl-sarcosine	8.0×10^{-10}	7.3×10^{-12}
(c) Other Compounds		
Asparagine 18°	0.7×10^{-9}	0.7×10^{-12}
Creatine 20°		8 ×10 ⁻¹²
Creatinine 20°		5.3×10^{-10}
Ornithine	1.7×10^{-11}	4.5 ×10 ⁻⁶
		8.7×10^{-13}
Taurine	1.8×10 ⁻⁹	3 ×10 ⁻¹³

(Kirk, P. L., and Schmidt, C. L. A., Univ. of Calif. Pub. Physiol., 7, 57, 1929; Scudder, H., Conductivity and Ionization Constants of Organic Compounds, New York, 1914.)

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ADDENDA: The reader should also consult Clark, H., in Gilman, H., Organic Chemistry, Vol. II, New York, p. 859 for the material of this chapter. Clark's review appeared after this chapter was written.

CHAPTER VI

PEPTIDES, PEPTIDASES, AND DIKETOPIPERAZINES

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1. INTRODUCTION

When a solution of acid, alkali, or proteolytic enzyme acts upon a protein, a decrease in the total mass of water in the solution occurs, and the final products of the reaction are a mixture of α-amino acids. The manner of linkage of these amino acids in the protein and the fate of the water must ultimately depend upon the nature of the reactive groups of the amino acids themselves. In addition to the α -amino and the α -carboxyl groups common to all the amino acids, there exist in certain of them other reactive radicals such as the guanidine nucleus in arginine, the ϵ -amino group in lysine, the imidazole ring in histidine, the γ -carboxyl group in glutamic acid, etc. Numerous investigations (1, 2, 3, 4, 5, 6) have proven that these extra groups of the trivalent amino acids exist free and uncombined when the acids form part of the protein molecule. This leaves only the α -amino and the α -carboxyl groups of the simple and of the complex amino acids to be considered as comprising those reactive groups whose combination in some fashion results in the protein molecule.

The nature of this combination has been investigated by the method of titration during the action of proteolytic enzymes on a protein substrate. It has been satisfactorily demonstrated by several investigators (7, 8, 9) that the action of pepsin and of trypsin on various proteins results always in the simultaneous liberation of one amino group and one carboxyl group. The only type of linkage which as far as we know, can yield, by hydrolytic splitting, an amino and a carboxyl group is an amide type of linkage, -CONH-. Thus -CONH-+HOH--COOH+-NH2. Therefore the protein molecule may in general be conceived as consisting of

¹ That the action of erepsin on certain proteins results in the liberation of more carboxyl groups than amino groups may be ascribed to the presence of considerable amounts of proline and oxyproline in these proteins (7).

 α -amino acids bound to each other through condensation of the α -amino group of one acid with the α -carboxyl group of the adjacent acid, this amide type of linkage being usually referred to as the peptide linkage.

The final confirmation of the presence of the peptide bond in the proteins is afforded by the action of tryptic and ereptic enzymes on synthetic combinations of amino acids bound in peptide linkage. The proteolytic enzymes of pancreatic and intestinal origin hydrolyze the synthetic peptide bond as well as the bond which occurs in native proteins. Therefore the latter linkage must be identical with the former on the assumption of the unique specificity of enzyme action.²

It had been recognized by many of the early workers that the action of the three proteases, pepsin, trypsin, and erepsin, on the same protein substrate was different in each case. The action of pepsin proceeds only to a certain degree and then ceases. The addition of fresh pepsin has no effect. If trypsin be added the reaction proceeds anew but finally slows down and ceases. However when erepsin is added to this mixture a rapid onset of hydrolysis is noted, the reaction finally coming to a standstill when only amino acids remain. The action of the three classes of enzymes is clearly different in each case and may reasonably be explained by the hypothesis that peptide linkages in different parts of the protein molecule are attacked by different enzymes. When the particular linkages available for each enzyme have been hydrolyzed, further action of the enzyme is without effect. In view of the enormous complexity of the protein molecule it is manifestly impossible at present to determine with any degree of certainty either the position of the linkages available for each enzyme or the nature of the amino acids participating in these linkages. The problem must be attacked from another point of view, namely, by the chemical synthesis of protein models. Large numbers of molecules of amino acids bound in peptide form must be studied. These peptide molecules necessarily must be as diverse as possible and contain a large variety of amino acids, both complex and simple, in as many configurations as the methods of synthesis permit. The treatment of such peptide molecules with the various proteolytic enzymes will afford a means of determining the molecular configuration conditioning the specific action of each type of enzyme. Such a pro-

² The apparent ability of pepsin to hydrolyze peptide bonds in the protein but not in the synthetic molecules is discussed later.

cedure will serve the double purpose of throwing light on both the probable configuration of the enzyme molecule and of the protein substrate.³

The primary step toward the realization of this problem therefore consists in devising methods for the synthesis of such peptide molecules.

2. THE SYNTHESIS OF PEPTIDES

We may consider the case of two molecules of amino acids, $R \cdot CH(NH_2)_A \cdot (COOH)_B$ and $R \cdot CH(NH_2)_C \cdot (COOH)_D$, which are to be combined through the peptide condensation of groups B and C to form the molecule $R \cdot CH(NH_2)_A \cdot CONH \cdot CHR \cdot (COOH)_D$. Only groups B and C are to be reactive which implies the necessity of suppressing the reactive character of groups A and D. This can be accomplished by appropriate substitution of these groups by such means as to neutralize the basic character of the former and the acidic character of the latter. These masking substituents must, however, be of such a nature that following the peptide condensation they may be easily removed from the groups A and D of the peptide molecule without any too great breakdown of the molecular structure or formation of side-reactions.

The masking of the carboxyl group D presents a very simple problem and may be solved in either of two ways depending on whether it is desired to perform the peptide synthesis in aqueous or in non-aqueous solution. In the former case, the acidic character may be neutralized by the formation of the alkali salt, i.e., neutralization with an equivalent amount of alkali. For working in non-aqueous solvents such as ether or chloroform, the carboxyl group must be converted into an ester. When it is desired to regenerate the carboxyl group in the peptide molecule it is only necessary to neutralize with acid in the former case or to saponify with alkali in the latter.

In contrast to the ease of treating the carboxyl group D, the satisfactory masking of the amino group A and the subsequent removal of the masking agent have presented great difficulties. In addition to choosing a masking group which will satisfy the criterion of easy removal, this agent must likewise have no effect upon the optical activity of the amino acid in which it is substituted.

³ Such synthetic molecules serving as protein models may be used to study the physico-chemical properties of the protein.

When groups A and D have been adequately masked the problem of the condensation of groups B and C to form the peptide bond arises. This has been answered for the most part by converting the carboxyl group B into the corresponding acid chloride or azide and allowing the latter to react with the unprotected amino group C of the second molecule. In either case HCl or N₃H is evolved and therefore an excess of alkali or of ester must be present if the reaction is to proceed.

$$\begin{aligned} &R \cdot COCl + NH_2 \cdot R \rightarrow R \cdot CONH \cdot R + HCl \\ &R \cdot CO(N_3) + NH_2 \cdot R \rightarrow R \cdot CONH \cdot R + N_3H \end{aligned}$$

In analogy with the saccharides, molecules containing two amino acids are called dipeptides; molecules with three amino acids in the chain are called tripeptides, etc. The term "polypeptide" is loosely used to designate any molecule higher than a dipeptide.

3. SURVEY OF THE METHODS OF PEPTIDE SYNTHESIS

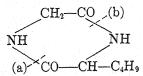
(1) The Hydrolysis of Diketopiperazines. The preparation of the first peptide dates from 1901 when Fischer and Fourneau (10) heated glycine anhydride with concentrated HCl to obtain glycylglycine:⁴

$$\begin{array}{ccc} \mathrm{NH} \cdot \mathrm{CH}_2 \cdot \mathrm{CO} & \mathrm{H}_2\mathrm{O} \\ | & | & \longrightarrow \\ \mathrm{CO} \cdot \mathrm{CH}_2 \cdot \mathrm{NH} & & \longrightarrow \end{array} \mathrm{NH}_2 \cdot \mathrm{CH}_2 \cdot \mathrm{CONH} \cdot \mathrm{CH}_2 \cdot \mathrm{COOH}$$

This method of hydrolytically splitting the anhydride ring by means of acid or alkali was extended by Fischer with varying degrees of success to anhydrides involving other amino acids. The ease of hydrolysis is determined by the character of the amino acids participating in the ring, those acids with long hydrocarbon chains forming extremely stable rings (11). The method moreover is limited, being obviously restricted only to the preparation of dipeptides and to such dipeptides derived from available diketo-

In point of historic fact, Curtius in 1882 (78) had obtained by the action of benzoyl chloride on silver glycinate, among other products, the N-substituted peptide, benzoylglycylglycine, $C_6H_6 \cdot CONH \cdot CH_2 \cdot CONH \cdot CH_2 \cdot COOH$. This synthesis led Curtius and Fischer into a sharp controversy over the question of priority and induced the former to resume his work on the preparation of a number of benzoylated peptides (15). It is worthy of note, however, that all of the Curtius products were of the N-substituted variety in none of which the amino group was free. Attempts to remove the benzoyl radical in order to regenerate the amino group always led to a complete hydrolysis of all the peptide bonds in the molecule.

piperazines. If a heterogeneous anhydride molecule which contained two different amino acids were to be selected, the hydrolytic products would be a mixture of two dipeptides whose separation would present considerable difficulties. Thus glycyl-leucine anhydride yields on hydrolysis both glycyl-leucine and leucyl-glycine (12).



- (a) Splitting to yield glycyl-leucine
- (b) Splitting to yield leucyl-glycine
- (2) The Ester Condensation. The esters of the mono- α -amino acids and dipeptides under various degrees of heating pass over readily into the diketopiperazine form.

$$\begin{array}{c} \text{NH} \cdot \text{CHR} \cdot \text{CO} \\ 2\text{R} \cdot \text{CH}(\text{NH}_2) \cdot \text{CO}(\text{OCH}_3) {\longrightarrow} \big| & \big| \\ \text{CO} \cdot \text{CHR} \cdot \text{NH} \end{array}$$

On the other hand, the presence of a β -amino group, as in α -, β -diaminopropionic acid and in isoserine, or an ϵ -amino group, as in lysine, results in the formation of the dipeptide ester with liberation of alcohol when the ester of the acid is heated (11). The cause of this behaviour, as Fischer pointed out, may well lie in the probability of the β - or ϵ -amino groups participating in the condensation and thus making the formation of the six-membered anhydride ring impossible. The reaction is indicated by:

$$NH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CO(OCH_3) {\longrightarrow}$$

$$NH_2 \cdot CH_2 \cdot CH(NH_2) \cdot CONH \cdot CH_2 \cdot CH(NH_2) \cdot COOH + CH_3OH$$

The considerable tendency of the amino acid and peptide esters to condense with the splitting off of alcohol is exhibited also in the case of the esters of the higher peptides (13, 14). Thus the tripeptide ester of glycine on heating passes into the corresponding hexapeptide ester from which the free hexapeptide may be obtained on saponification:

$$\begin{split} 2\mathrm{NH_2}\cdot\mathrm{CH_2}\cdot\mathrm{CONH}\cdot\mathrm{CH_2}\cdot\mathrm{CO(OCH_3)} & \longrightarrow \\ \mathrm{CH_3OH} + \mathrm{NH_2}\cdot\mathrm{CH_2}\cdot\mathrm{CO(NH}\cdot\mathrm{CH_2}\cdot\mathrm{CO)_4NH}\cdot\mathrm{CH_2}\cdot\mathrm{CO(OCH_3)} \end{split}$$

This method is restricted only to tripeptides of the simple monoamino and monocarboxylic acids. It proved extremely useful in the synthesis of the large, long-chained polypeptides which Fischer and Abderhalden later undertook.

(3) The Use of Halogenacyl Compounds. The two methods described above, while useful in certain restricted cases, nevertheless do not permit of a general method of peptide synthesis whereby any desired amino acid can be placed in any position in the peptide combination. Curtius (15) had shown, by the use of the N-benzovl amino acid azide, that the peptides could be built up, one acid at a time, to any desired length. However, the difficulty in the final removal of the N-benzovl radical from the peptide without breaking the peptide linkages proved insurmountable. Fischer solved this problem by the ingenious device of starting with the corresponding α -halogen acid (16) rather than with the corresponding α-amino acid. Treatment of the α-halogen acid with PCl₅ converts it into the acid chloride which then easily couples with the free amino group of any amino acid or peptide with the liberation of HCl. The reaction must therefore proceed either in an excess of alkali if an aqueous medium is chosen or else in the presence of an excess of free amino acid or peptide ester if an ether or chloroform solution is desired. The reaction may be generalized as follows:

> $R_1 \cdot CHX \cdot COCl + R_2 \cdot CH(NH_2) \cdot COOH \rightarrow$ $R_1 \cdot CHX \cdot CONH \cdot CH(R_2) \cdot COOH + HCl$

The halogenated peptide may then be converted into the corresponding free peptide by treating it under various conditions with

an excess of ammonia:

 $R_1 \cdot CH(NH_2) \cdot CONH \cdot CH(R_2) \cdot COOH + NH_4X$

The halogenated acyl chlorides which Fischer employed in the synthesis of a large number of peptide molecules are listed below together with the amino acid residues to which they give rise following treatment with ammonia (17):

Choroacetyl chloride —Glycyl α-Bromopropionyl bromide —Alanyl

α-Bromobutyryl chloride —α-Aminobutyryl

α-Bromoisocaproyl bromide —Leucyl

α-Bromohydrocinnamyl chloride—Phenylalanyl

Higher peptides may be prepared in stepwise fashion by treating the halogenated peptide with PCIs and coupling the resulting acid chloride with a free amino acid in alkaline solution or else more rapidly by coupling the acid chloride with still another peptide. Perhaps no better illustration of the combination of this method together with methods 1 and 2 can be offered than by Fischer's synthesis (18) of the octadecapeptide, leucyl-triglycyl-leucyltriglycyl-leucyl-octaglycyl-glycine: the hydrolysis of glycine anhydride yields glycyl-glycine (method 1) which, coupled in alkaline solution with chloroacetyl chloride (method 3), yields, after treatment with NH₃, the tripeptide diglycyl-glycine. Condensation of the ester of the tripeptide yields the ester of the hexapeptide pentaglycyl-glycine (method 2) from which the ester group may be removed by saponification. α-Bromoisocaproyl chloride is then coupled with diglycyl-glycine, yielding α-bromoisocaproyl-diglycylglycine. The acid chloride of the latter coupled with the hexapeptide of glycine yields α-bromoisocaproyl-octaglycyl-glycine which by means of NH₃ yields leucyl-octaglycyl-glycine:

 $\mathbf{NH_2 \cdot CH(C_4H_9) \cdot CO(NH \cdot CH_2 \cdot CO)_8NH \cdot CH_2 \cdot COOH}$

In similar fashion this decapeptide may be coupled with bromoiso-caproyl-diglycylglycine, the product following treatment with NH₃ yielding the tetradecapeptide, leucyl-triglycyl-leucyl-octaglycyl-glycine:

$$\begin{split} \mathbf{NH_2 \cdot CH(C_4H_9) \cdot CO(NH \cdot CH_2 \cdot CO)_3NH \cdot CH(C_4H_9) -} \\ \mathbf{CO(NH \cdot CH_2 \cdot CO)_8NH \cdot CH_2 \cdot COOH} \end{split}$$

Repetition of the above reaction with bromoisocaproyl-diglycyl-glycine finally yields the octadecapeptide, leucyl-triglycyl-leucyl-triglycyl-leucyl-octaglycyl-glycine:

$$\begin{split} \mathbf{NH_2} \cdot \mathbf{CH}(\mathbf{C_4H_9}) \cdot \mathbf{CO}(\mathbf{NH} \cdot \mathbf{CH_2} \cdot \mathbf{CO})_3 \mathbf{NH} \cdot \mathbf{CH}(\mathbf{C_4H_9}) - \\ \mathbf{CO}(\mathbf{NH} \cdot \mathbf{CH_2} \cdot \mathbf{CO})_3 \mathbf{NH} \cdot \mathbf{CH}(\mathbf{C_4H_9}) \cdot \mathbf{CO}(\mathbf{NH} \cdot \mathbf{CH_2} \cdot \mathbf{CO})_8 \mathbf{NH} \cdot \mathbf{CH_2} \cdot \mathbf{COOH} \end{split}$$

This eighteen-membered polypeptide resembled a protein in that it gave the biuret reaction and was readily "salted out" of solution.

It is evident that the preparation of fully optically-active peptides by the method of the halogenated acyls is fraught with difficulty. The halogenation of a fatty acid through substitution of an α -hydrogen leads to a racemic compound, the separation of whose isomers is quite tedious. In the endeavor to prepare an op-

tically active halogenated acid through action of nitrosyl halide on an optically active amino acid, Fischer (19) made the disappointing discovery that a Walden inversion invariably occurs. Thus the action of nitrosyl bromide on l-alanine results in the formation of d- α -bromopropionic acid. In this fashion the peptides of the antipodes of the naturally-occurring amino acids could be prepared but this was hardly satisfactory. In order to arrive at the l-halogen acids, Fischer was led to the preparation of the optical antipodes of the naturally-occurring amino acids through resolution of the racemic acids. The action of nitrosyl bromide on d-alanine leads to the formation of l-bromo propionic acid which can be converted to optically active l-alanyl-l-valine (20): d-alanine NOBr l-bromopropionic acid PCl_5 l-bromopropionyl chloride +l-valine $\rightarrow l$ -bromopropionyl-l-valine NH_3 l-alanyl-l-valine.

In spite of Fischer's device to prepare the optically-active peptides, it is doubtful if a certain amount of racemization did not occur. Particularly in the step involving the amination of the position occupied by the halogen—in the case of certain peptides often requiring high temperatures—a partial racemization could conceivably take place.

Besides the practical difficulties in the way of resolving the optical isomers of the amino acids and the dangers of subsequent racemization, the halogenated acyl method is likewise limited in its applicability. It can be used only with the simple monoamino acids and thus even Fischer's octadecapeptide is but an elongated dipeptide with one amino and one carboxyl group. The halogenacyl method cannot be used to introduce the hexone bases into peptides, and yet it is just such complex amino acids which form the reactive groups of proteins. Again in certain instances the treatment of the halogen peptide with excess of ammonia sometimes results not in a substitution of amino for halogen, but in hydrolysis, the product being a hydroxy peptide amide. Thus bromoisocaproyl-proline on treatment with NH₃ yields not leucyl-proline but oxyisocaproyl-proline amide (21).⁶ Even in the in-

⁶ The action of NH₂ on the β -phenyl- α -bromopropionyl (phenylalanyl) residue sometimes leads to the formation of cinnamyl derivatives owing to the splitting off HBr (79).

⁵ Throughout this chapter the naturally-occurring amino acids which possess the same spatial configuration and belong to the *l*-family will be designated by the *l*-configuration. Their optical antipodes will then be referred to as *d*-compounds regardless of their optical behaviour.

stances where amination takes place it is probable that the yield of peptide is often diminished by this reaction. Furthermore, the final purification of the peptide is rendered difficult because of the presence of considerable amounts of ammonium halide. The last traces of the salt can be separated from the crystalline peptide only by many crystallizations.

Bertho and Maier (22) devised another method of substituting the α -halogen by NH₂. It consists in treating the halogenated peptide ester with sodium azide, thus replacing the halogen with the azido group. On catalytic hydrogenation the latter is reduced to the amino group. Subsequent saponification of the ester, followed by acidification, yields the free peptide:

 $R \cdot CH(Br) \cdot CONH \cdot CHR \cdot CO(OC_2H_5) + NaN_3 \rightarrow$

 $R \cdot \mathrm{CH}(\mathrm{N_3}) \cdot \mathrm{CONH} \cdot \mathrm{CHR} \cdot \mathrm{CO}(\mathrm{OC_2H_5}) + \mathrm{NaBr}$

 $R \cdot CH(N_3) \cdot CONH \cdot CHR \cdot CO(OC_2H_5) + H_2 \rightarrow$

 $R \cdot CH(NH_2) \cdot CONH \cdot CHR \cdot CO(OC_2H_5) + N_2$

This method proved of value in cases where the use of aqueous ammonia on the halogenated molecule failed to effect the formation of the free peptide. Its limitations, however, are similar to those of the Fischer method.

(4) The Azlactone Method. In the endeavor to extend the scope of peptide synthesis Bergmann and his co-workers made use of the properties of the azlactones and thus devised a method whereby the more complex amino acids might be brought in peptide combination. The principle of the method rests on the reaction first described by E. Erlenmeyer and Frustück (23) in which the aromatic aldehydes condense with N-acylated glycine in the presence of a dehydrating agent to form the azlactone of the corresponding $\Delta\alpha\beta$ -acid.

$$\begin{array}{c} C_6H_5 \cdot C - O \\ C_6H_5 \cdot CHO + C_6H_5 \cdot CONH \cdot CH_2 \cdot COOH - \longrightarrow \\ C_6H_5 \cdot CH = C - C = O \end{array}$$

Bergmann, Stern and Witte (24) found that the azlactone, while relatively stable toward cold water, will, however, react energetically with amines. The reaction of the azlactone with the alkaline salt of an amino acid results in the opening of the azlactone ring

together with simultaneous coupling of the liberated carboxyl group with the free amino group of the second amino acid. The resulting compound is N-acylated $\Delta\alpha\beta$ -peptide. On catalytic hydrogenation followed by removal of the N-acyl radical, the dipeptide is formed. The method may be illustrated by the synthesis of phenylalanyl-glutamic acid (24):

$$C_6H_5 \cdot CH = C$$
 $COOH$
 $N = C(CH_3) - O + NH_2 \cdot CH \cdot CH_2 \cdot CH_2 \cdot COOH$

Azlactone of N-aceto-
amino-cinnamic acid

$$\begin{array}{c} \text{COOH} \\ \mid \\ \text{C}_6\text{H}_5 \cdot \text{CH} = \text{C}--\text{CONH} \cdot \text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}} \\ \mid \\ \text{NH} \cdot \text{CO}(\text{CH}_3) \\ \text{N-Acetoamino-cinnamyl-l-glutamic acid} \end{array}$$

$$\begin{array}{c} \text{COOH} \\ \mid \\ \text{C}_6\text{H}_5 \cdot \text{CH}_2 \cdot \text{CH} \cdot \text{CONH} \cdot \text{CH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH} \\ \longrightarrow \\ \text{NH} \cdot \text{CO(CH}_8) \\ \alpha\text{-N-Acetyl-l-phenylalanyl-l-glutamic acid} \end{array}$$

COOH
$$C_6H_5 \cdot CH_2 \cdot CH \cdot CONH \cdot CH \cdot CH_2 \cdot CH_2 \cdot COOH$$

$$NH_2$$

$$l\text{-Phenylalanyl-}l\text{-glutamic acid}$$

α-N-Acetyl-d-phenylalanyl-l-glutamic acid

d-Phenylalanyl-l-glutamic acid

The reduction of the double bond of the N-acetyl peptide leads to the formation of an asymmetric carbon atom with the subsequent production of a mixture of d- and l-forms which, in consequence of the presence of l-glutamic acid in the molecule, are not optical antipodes. The separation of these forms can be effected by crystallization.

By this method such peptides as d-phenylalanyl-l arginine (25), d-tyrosyl-l arginine (26) (by the use of the azlactone of α -N-acetoamino-p-acetoxy cinnamic acid), and, by an analogous method, d-l-histidyl-glycine (27)—were prepared. Although the

azlactone procedure led to the synthesis of peptides of some complex acids which the previous Fischer methods could not, the results as a whole were still far from satisfactory. The removal of the N-acetyl group by hot acid entailed a certain breakdown of the molecule but most serious of all, the separation of the d-,l-peptide mixture, as in the case of the arginine peptides cited above, led to the isolation only of the unnatural d-isomer of d-,l-phenylalanyl-l-arginine and of d-,l-tyrosyl-l-arginine. A general method for the synthesis of pure optically-active l-peptides still remained to be found.

(5) The Carbobenzoxy Synthesis. A further important step toward a general method of peptide synthesis whereby any amino acid, whether simple or complex, could be bound in peptide linkage was arrived at by Bergmann and Zervas (28), just thirty years after Fischer and Fourneau had prepared glycyl-glycine. In this method, the masking of the amino group A (see above) was accomplished by condensation of the benzyl ester of chlorocarbonic acid (carbobenzoxy chloride, C6H5 · CH2 · O · COCl) with the amino group by the Schotten-Baumann technique. The removal of this masking group is the essential step in this synthesis, for it can be split off quantitatively from the peptide by the use of catalytic hydrogen at room temperature, yielding simultaneously the regenerated amino group, carbon dioxide, and toluol.7 The method is quite powerful and, through the use of the carbobenzoxy technique, amino acids such as lysine, which hitherto had been unavailable for peptide synthesis, could now be employed.8 Insight into the nature of enzyme action, afforded by a diverse assortment of optically active and crystalline substrates was facilitated, and studies of the physical chemical properties of peptides containing more than one dissociated amino group and one dissociated carboxyl group could be undertaken (29). Through the study of the properties of lysyl-glutamic acid the physical chemistry of the peptides received a new impetus. As an example of the method, the synthesis of l-lysyl-l-glutamic acid may be cited (30).

⁷ It is interesting to note that Fischer had used both carbomethoxy chloride and carboethoxy chloride as N-acyl radicals; the removal of these groups provided some difficulty (80), (16).

⁸ The extraordinary reactivity of the amino groups of the lysine peptides toward nitrous acid is quite unusual and unexpected. Whereas lysine itself on treatment with HNO₂ gives up all of its amino nitrogen in not less than 15 minutes, the lysyl peptides yield theirs in about half this time (81, 82). On the other hand, the proteins may require several hours for deamination.

The end point of the hydrogenation can in all cases be easily ascertained by the cessation of the evolution of CO₂.

A significant extension of the method to the case of the N-carbobenzoxydicarboxylic acids such as aspartic and glutamic acids by Bergmann, Zervas, and Salzmann (31) provides a method of masking only the proximal carboxyl group and hence permits the distal carboxyl group to be used for peptide combination. This was accomplished by treating the anhydride of the N-carbobenzoxydicarboxylic acid,

$$\begin{array}{c} \mathrm{C_6H_5 \cdot CH_2 \cdot O \cdot CONH \cdot CH \cdot CO} \\ \\ \mathrm{O} \\ \\ \mathrm{(CH_2)_n - CO} \end{array}$$

with alcohol, the alcoholic radical yielding only the α -ester, the other carboxyl group remaining free and available for coupling,

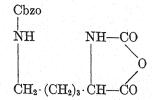
$$\begin{array}{c} \textbf{C}_{6}\textbf{H}_{5} \cdot \textbf{C}\textbf{H}_{2} \cdot \textbf{O} \cdot \textbf{CONH} \cdot \textbf{CH} \cdot \textbf{CO}(\textbf{OC}_{2}\textbf{H}_{5}) \\ \\ (\textbf{CH}_{2})_{n}\textbf{COOH} \end{array}$$

In this fashion glutamine and asparagine could be prepared as well as β -aspartyl and γ -glutamyl peptides. On the other hand, in the case of the diamino acids, such as lysine, Bergmann, Zervas, and Ross (32) devised a method whereby only the ϵ -amino group might be masked by the carbobenzoxy group, leaving the α -amino group free and available for peptide coupling. This consists in warming the acid chloride of α -, ϵ -dicarbobenzoxylysine,

$$\begin{array}{ccc} Cbzo & Cbzo \\ & & | \\ NH & NH \\ & | \\ CH_2 \cdot (CH_2)_3 \cdot CH \cdot COCl \end{array}$$

thereby forming the ϵ -carbobenzoxy- α -carboxylysine anhydride:

⁹ The general use of N-carbobenzoxy dicarboxylic acid anhydrides was first indicated by Bergmann and Zervas in their synthesis of glutamyl-glutamic acid and aspartyl-tyrosine (28). Here the anhydride is brought directly into reaction with the ester of the second amino acid. The uncertainty of this procedure is evident since it is sometimes unpredictable which one of the two carboxyl groups will enter into the peptide coupling. The remarkable influence of the solvent in determining which of the two carboxyl groups will participate in the linkage is described by Grassmann and Schneider (39).



The anhydride ring is readily opened by HCl, yielding ϵ -carbobenzoxy-lysine. These extensions of the general method permit the synthesis of tri- and higher peptides which contain the dicarboxylic acids or the diamino acids in the interior of the chain as they most probably occur in the proteins.

In only one instance does the method of removal of the carbobenzoxy group by means of catalytic hydrogenation fail, and that is in the case of cystine peptides. The removal of the N-carbobenzoxy group from this class of substances has, however, been accomplished by two procedures:

(a) Reduction in liquid ammonia by the use of metallic sodium by du Vigneaud and his associates (33). This is a powerful tool not only for this reaction but because various S- substituted ethers may be formed by replacing the sodium with alkyl or aryl halides.

(b) Reduction by the use of phosphonium iodide by Harington and Mead. This technique enabled these authors to perform the brilliant synthesis of optically-active glutathione (34).

The use of the two methods above to remove the carbobenzoxy group leads of course to the simultaneous reduction of the cystyl residue to cysteyl. The oxidation to the cystine peptide is, however, easily accomplished (33).

Table I lists the most representative of the group of complex peptides which the use of the carbobenzoxy technique has made possible.¹⁰

TABLE I Dipeptides Tripeptides Glycyl-l-proline (35) l-Cystyl-diglycine (33) Glutathione (34) l-Lysyl-l-glutamic acid (30) l-Glutaminyl-glycyl-glycine (41) l-Lysyl-l-histidine (30) α-Hippuryl-ε-carbobenzoxy-ll-Glutamyl-l-glutamic acid (28) l-β-Aspartyl-l-tyrosine (28) lysamide (32) l-Tyrosyl-l-tyrosine (36) Tetrapeptide Glycyl-l-alanyl-l-leucyl-l-glutamic Glycyl-l-arginine (37) Glycyl-l-glucosamine acid (38) acid (42) l-Tyrosyl-l-aspartic acid (36) l-Aspartyl-glycine (39) Glycyl-d, l-\alpha-aminotricarballylic acid (40)

¹⁰ Certain complex peptides recently synthesized include carnosine (Sifferd, R. H., and du Vigneaud, V., J. Biol. Chem., 111, 393 (1935)); isoglutathione (du

4. THE ISOLATION OF PEPTIDES FROM PROTEINS

From partially hydrolyzed proteins, several peptides have been isolated, thus providing an additional confirmation of the peptide structure of the proteins. Curiously enough, those which have been definitely characterized as chemical individuals through comparison with the corresponding synthetic peptides, have been mostly dipeptides. Table II lists those dipeptides which have been isolated, together with the protein which is their source.

TABLE II

Peptide	Protein Source
Glycyl-d-alanine (43)	Silk fibroin
Glycyl- <i>l</i> -leucine (44)	Elastin
Glycyl-l-tyrosine (45)	Silk fibroin
d-Alanyl-glycine (45)	Silk
d-Alanyl-l-leucine (43)	Elastin
l-Prolyl-l-phenylalamine (46)	Gliadin

The method of analysis of the constitution of these peptides first employed β -naphthalenesulfochloride, using the Schotten-Baumann technique. Hydrolysis of the N-acylated peptide yields a mixture whereby that amino acid which had possessed the free amino group in the peptide is now found as the N-naphthalenesulpho-substituted acid (43). Another method of determining the constitution of these peptides rests on the observation of the change in direction of optical rotation during hydrolysis of the peptide (47). While fairly successful in a few instances, the applicability of this procedure is evidently limited.

In one method of peptide analysis, devised by Bergmann and Zervas (42), the peptide is degraded one amino acid at a time from the carboxyl end of the molecule. The reactions involved may be described in general terms as follows:

$$\begin{array}{c} \text{C}_6\text{H}_5 \cdot \text{CONH} \cdot \text{CH}(\text{R}_1) \cdot \text{CONH} \cdot \text{CH}(\text{R}_2) \cdot \text{CO}(\text{OCH}_3) \\ \\ \text{N-Benzoylated peptide ester} \end{array}$$

 $C_6H_5 \cdot CONH \cdot CH(R_1) \cdot CONH \cdot CH(R_2) \cdot CO(NHNH_2)$ N-Benzoylated peptide hydrazide

Vigneaud, V., Loring, H. S., and Miller, G., J. Biol. Chem. 118, 391 (1937)); l-cysteyl-l-cysteine and l-cystyl-l-cystine (Greenstein, J. P., J. Biol. Chem., 121, 9 (1937)); l-cystyl-diglycine and l-aspartyl-l-histidine (Greenstein, J. P., unpublished data). By means of an improved synthetic procedure, glutathione has become available in quantity (du Vigneaud, V., and Miller, G., J. Biol. Chem. 116, 469 (1936)).

$$\begin{array}{c} C_6H_5\cdot CONH\cdot CH(R_1)\cdot CONH\cdot CH(R_2)\cdot CO(N_3) \\ \hline N-Benzoylated \ peptide \ azide \end{array}$$

 $\begin{array}{c} \text{H}_2\\ \text{C}_{\scriptscriptstyle{\theta}}\text{H}_{\scriptscriptstyle{\delta}}\cdot \text{CONH}\cdot \text{CH}(\text{R}_1)\cdot \text{CONH}\cdot \text{CH}(\text{R}_2) \text{CONH}(\text{OCH}_2\cdot \text{C}_{\scriptscriptstyle{\theta}}\text{H}_{\scriptscriptstyle{\theta}}) \\ \text{N-Benzoylated peptide benzyl urethane} \end{array}$

 $\begin{array}{c} \text{C}_6\text{H}_5 \cdot \text{CONH} \cdot \text{CH}(\text{R}_1) \cdot \text{CONH}_2 + \text{HCO}(\text{R}_2) + \text{CO}_2 + \text{NH}_3 + \text{C}_6\text{H}_5 \cdot \text{CH}_3 \\ \text{N-Benzoyl acid amide} \end{array}$

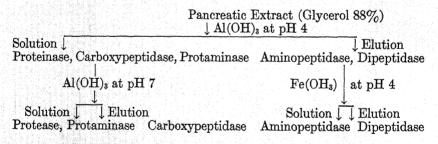
The amino acid which has been split off from the chain appears therefore as a readily identifiable aldehyde containing one less carbon atom. In the case of long chain peptides, the residual N-benzoylated peptide amide may be treated further with hydrazine to form the hydrazide. The process can then be repeated.

A very satisfactory method for the determination of the configuration of the peptides, and in time presumably the proteins, rests in the specific action of the peptidases from the pancreas and the intestines, and other sources such as yeast, which act in a definite manner at specific points on linkages in the peptide molecule.

5. THE PEPTIDASES

Extensive research into the methods of synthesizing peptides led to the preparation of a large number of diverse molecules, many of which are detailed on the previous pages. Whether these synthetic peptides could be attacked by the proteolytic enzymes of the digestive tract remained the criterion of the occurrence of such structures in native proteins. Inasmuch as Fischer had prepared the first molecules of this type he, together with Abderhalden, were the earliest workers to investigate their behaviour with enzymes. Unfortunately, this pioneer work led to results which were quite inconclusive, for peptides of almost similar constitution behaved quite differently toward the same enzyme. It was soon recognized that, whereas the synthetic peptide substrates of Fischer were quite pure, the enzyme materials which he employed were grossly impure, being in the main crude extracts of the minced digestive organs. The early workers were hampered, moreover, by inadequate analytical methods. The problem was clarified when Sörensen defined the effect of pH on the activity of enzymes, and Willstätter began the purification and isolation of homogeneous enzyme materials. It soon became apparent, through the purification by selective adsorption on aluminum and iron gels, that the crude extracts of the pancreas and the intestines consisted not of trypsin and erepsin, respectively, but of tryptic and ereptic complexes, each of which consists of a number of enzymes. Each member of these groups of enzymes was characterized by their being able to attack the peptide molecule in a definite manner, hydrolyzing linkages at specific positions in the peptide chain. By the use of peptides of known constitution and by the subsequent procedure of analyzing for the split products of the enzymic reaction it can be ascertained where these hydrolyzable linkages occur. In this fashion it was discovered that a specific enzyme carboxypeptidase can be isolated from pancreatic extract which splits off amino acids from the carboxyl end of the peptide chain (48, 49). Again. a specific enzyme, aminopeptidase, can be isolated from the intestinal mucosa which splits off that amino acid which carries the amino group from the peptide chain (50). Still another enzyme was found that attacks only dipeptides, (50), etc.

By the method of fractional adsorption of crude organ and plant extracts at various pH values, using various colloidal gels, and testing the enzyme fractions with peptides of known constitution, the specific effect, if any, of each fraction can be ascertained and enzymes of individual specificity can be isolated. A typical example of the fractionation of pancreatic extract is the following (51):



The specific behaviour of the peptidases is illustrated by the data which are given in Table III. These are taken largely from the work of Bergmann, et al. (52).

Table III Specificity of Carboxypeptidase

Specificity of Carooxi	s pepiraase
Substrate	Hydrolysis
Glycyl-glycine	
Chloroacetyl-tyrosine	+
Chloroacetyl-N-methyltyrosine	
d,l-Leucyl-glycyl-l-tyrosine	+ (l-Tyrosine split off)
Chloroacetyl-l-tyrosine ester	

From the data which are given in Table III, the following conclusion as to the specificity of the enzyme carboxypeptidase may be drawn:

- 1. The presence of a free amino group on the same C-atom as the peptide bond, inhibits hydrolysis.¹¹
 - 2. The presence of a free terminal carboxyl group is essential.
- 3. Substitution of the hydrogen of the peptide linkage precludes hydrolysis.
- 4. The peptide linkage nearest to the carboxyl group in a polypeptide is attacked.

This type of determination, using specially constructed peptide substrates, may be extended to the study of the specificity of other

TABLE IV

Enzyme	Substrates (Either a-NH2 c	or α-COOH or both are required)
	Соон	
Carboxypeptidase	X·CONH·CH R	(R=H or alkyl radical)
	X	
Aminopeptidase	NH2 · CH · CONH · CH	(R = H or alkyl radical)
	R R	
	Соон	
Dipeptidase	NH2 · CH · CONH · CH	(R=H or alkyl radical)
	RRR	
Prolidase	$NH_2 \cdot CH \cdot CON : P \cdot COOH$	(R=H or alkyl radical)
	R	(P=Proline residue)
Protaminase	Y-Protamin-COOH	(Y = free or substituted NH ₂)
		(X = Balance of molecule containing -COHN-etc. groups)

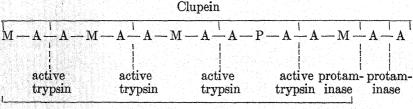
peptidases and some proteases from both animal and plant sources. In this fashion, the specificity of animal (53, 54) and plant (55, 56, 57) amino peptidase and dipeptidase, papain (58, 59), and

¹¹ Bergmann, Zervas, Salzmann and Schleich (36) have found that, whereas the free NH₂-containing peptides are not attacked by carboxypeptidase, *l*-tyrosyl-*l*-tyrosine is observably hydrolyzed. In general, the presence of a phenyl radical in the peptide molecule has an accelerating action on this enzyme.

protaminase (60) was discovered.¹² Table IV lists the structures which are attacked by each type of peptidase:¹³

Conversely, when the specificity of each individual enzyme is known, the elucidation of the constitution of peptide molecules of unknown structure may be accomplished. Although new, this type of investigation is one of the most promising for determining the structure of complex polypeptides and even the proteins. Two excellent examples of this method of peptidase analysis may be cited:

- (a) The structure of glutathione by Grassmann and co-workers (61): Action of carboxypeptidase on glutathione results in the splitting off from the tripeptide of a molecule of glycine in nearly quantitative amount. This is incontrovertible evidence that glycine occupies the end position in the chain and carries the terminal carboxyl group.
- (b) The structure of the protamin, clupein, by Waldschmidt-Leitz (62): This study of the complex molecule of a protamin has served not only to elucidate the structure of the protein, but also the probable specific action of active trypsin protease. The mode of action of this enzyme, together with that of protaminase, on the molecule of clupein may be represented as follows:



Clupeon

where M refers to any one of four different monoamino acids, P to proline, and A to arginine. The total number of amino acids in clupein is fifteen, of which ten are arginine. Protaminase splits off two terminal arginine molecules, yielding clupeon. Active trypsin protease then attacks the latter, yielding two dipeptides and three tripeptides, the constitution of which is ascertained by the specific action of dipeptidase on the dipeptides, and of aminopolypeptidase

¹² Although it had been previously supposed that peptides containing the optical antipodes of the naturally-occurring amino acids could not be attacked by peptidases, Bergmann, Zervas, Fruton, Schneider, and Schleich made the interesting discovery that peptides of d-alanine can be slowly hydrolyzed by dipeptidase (53). The effect of the optical configuration therefore, is solely upon the rate of the reaction.

¹³ An enzyme, prolidase, has been found in the intestinal tract which splits the -CON = linkage (Bergmann, M., and Fruton, J. S., J. Biol. Chem. 117, 189 (1937)).

and carboxypeptidase on the tripeptides, respectively. The results indicate that the two dipeptides have the structure A-M and M-A, and that the three tripeptides have the structures A-M-A, A-M-A, and A-P-A. The action of active trypsin protease is therefore confined to A-A linkages of the protamin molecule. Knowing the constitution of the split products, that of the original molecule may be inferred. The method, as is evident, is capable of considerable extension to other complex polypeptides and proteins.^{14,15}

No synthetic polypeptide yet prepared has been found to serve as a substrate for pepsin. However this enzyme undoubtedly splits -CONH-linkages as the hydrolytic ratio, $\frac{\text{COOH groups liberated}}{\text{NH}_2 \text{ groups liberated}} = 1, \text{ found for several proteins, indicates}$

NH₂ groups liberated (8, 9). The substrate molecule must, however, be of considerable size and complexity, inasmuch as even the protamins are not at-

tacked by pepsin.16

6. THE SYNTHETIC ACTION OF PROTEASES

If the reaction of enzymes on proteins by enzymes be reversible, then by suitably altering the concentration of the medium, the split-products of the reaction, in the presence of the enzyme, should resynthesize to form the original molecule. The problem of enzymic synthesis was first approached by Croft-Hill in the synthesis of iso-maltose by the action of maltase on a concentrated solution of glucose. The enzymic synthesis of proteins was undertaken by numerous investigators, among the earliest of whom were Robertson (63) and Taylor (64). More recently the problem of peptic synthesis has been handled in a comprehensive manner by Wasteneys and Borsook (65).¹⁷ The extent of protein synthesis has

¹⁵ See Linderström-Lang (51) for an excellent discussion of the application of

enzymes to the elucidation of protein structure.

¹⁶ Gurin and Clarke (*J. Biol. Chem.*, **107**, 395 (1934)) found that the benzene sulfonation of gelatin, blocking the free amino groups of lysine, inhibits its digestion by pepsin.

¹⁷ An interesting and significant application of the method applied to the peptic synthesis of digested thyroglobulin has been made by Salter (84). The physiologically inactive iodine-containing fraction of the protein resulting from peptic digestion was resynthesized by pepsin to form a physiologically-active complex which will relieve myxedema. Whether pepsin may synthesize the O-ether linkage characteristic of thyroxine is a fascinating question.

¹⁴ From a knowledge of the specificity of the peptidase and the particular configuration of the peptide substrate which it attacks, hypotheses of the probable configuration of the peptidase itself may be advanced. In a series of interesting papers (83) Bergmann and his co-workers have considered this question.

been measured by the decrease in amino nitrogen and the increase in the trichloracetic acid-precipitability of the partially resynthesized products. Whether the synthesis by means of pepsin and trypsin involves the combination of free amino and carboxyl groups into normal peptide linkages has as yet not been definitely determined. A promising line of investigation might be concerned with the synthesis of simple peptides by the peptidases. It would be of great importance to ascertain whether an enzymically-synthesized peptide could be hydrolyzed by the same enzyme which synthesized it. An indication that the peptide linkage may be enzymically synthesized is afforded by the work of Krebs (66) on the amide-splitting enzyme of the kidney.¹⁸

7. THE DIKETOPIPERAZINES

That the protein exists for the greater part, if not entirely, in the form of polypeptide chains has been adequately demonstrated. The question has often been raised, however, whether there may not be certain cyclic configurations within the molecule, of which the most probable would be the diketopiperazines.

This class of substances belongs to the six-membered heterocyclic systems in which the ring encloses two peptide linkages. It consists in the combination of two amino acids with the loss of the elements of two molecules of water between the two pairs of α -amino and carboxyl groups.

$$\begin{array}{ccc} 2R \cdot CH(NH_2) \cdot COOH \rightarrow & NH \cdot CHR \cdot CO + 2H_2O \\ & & | & | \\ & CO \cdot CHR \cdot NH \end{array}$$

The first known member of this class, the so-called leucinimide, was obtained by Bopp in 1849 (67):

$$C_4H_9 \cdot CH \cdot NH \cdot CO$$

 $CO \cdot NH \cdot CH \cdot C_4H_9$

There are three general methods for the preparation of the diketopiperazines:

- (a) Heating the amino acid in a stream of CO₂ or HCl: In this fashion the anhydrides of leucine, phenylanine, and sarcosine were prepared.
 - (b) Conversion of the amino acid ester: Curtius and Goebel (68)

¹⁸ Since the above was written, Bergmann and Fraenkel-Conrat (*J. Biol. Chem.*, **119**, 707 (1937)) performed the first unequivocal peptide synthesis through the action of papain on a mixture of hippuric acid and aniline. In the presence of cysteine, hippuryl-anilide was formed.

found that glycine ester in aqueous solution spontaneously passes over into the corresponding glycine anhydride. The rate of conversion depends on the ester radical, being greatest for the methyl ester, and progressively decreasing in the case of the higher alkyl esters (69). The larger-chained amino acid esters require heating for their transformation into the anhydride form (11). The diketopiperazines of histidine and lysine were prepared in this way.

(c) Conversion of the dipeptide ester: The above two methods lead to homogeneous molecules in which the ring consists of only one kind of amino acid. To prepare the mixed anhydrides, the esters of the mixed dipeptides must be employed. The condensation of the latter is by far the simplest method of preparing the diketopiperazine, the dipeptide ester in alcoholic-NH₃ solution passing rapidly into the anhydride form even at 0°. Not only the free dipeptide ester may be employed in the synthesis, but also the α -halogenated-acyl-peptide ester. Thus Fischer and Otto (16), on treating chloroacetylalanine ester with alcoholic NH₃ in the cold, obtained the first mixed diketopiperazine:

$$\begin{array}{c} \mathrm{CICH_2 \cdot CONH \cdot CH(CH_3) \cdot CO(OC_2H_5) + 2NH_3 \rightarrow} \\ \mathrm{CH_2 - CO} \\ \mathrm{NH_4Cl + C_2H_5OH + HN} \\ \mathrm{CO - CH} \\ \mathrm{CH_3} \end{array}$$

The marked tendency of the dipeptide esters to pass almost spontaneously into the diketopiperazine is a characteristic of this class of substances and differentiates it sharply from the case of the amino-acid esters which, with the exception of glycine, pass with some difficulty into the anhydride form.¹⁹ The dipeptides too,

¹⁹ The ease of conversion of the dipeptide ester permits the formation of optically-active diketopiperazines from active peptides. The configuration of the anhydrides of two active amino acids permits the formation of two optically active cis-forms and one optically-inactive trans-form. Thus *l*-alanyl-*l*-alanine ester yields the optically-active *l*-alanine anhydride which has the cis-configuration; *d*-alanyl-*l*-alanine ester as well as *l*-alanyl-*d*-alanine ester yield the inactive trans-form of the anhydride. This may be generalized as follows:

show great tendency to pass over into diketopiperazines (70) at higher temperatures. This latter fact must be considered, when, following the isolation of diketopiperazines from protein digests, their occurrence in the original protein molecule is postulated.

8. THE ISOLATION OF DIKETOPIPERAZINES FROM PROTEINS

From the various protein digests several anhydrides have been identified (see Edlbacher (71) for a list of these). Whether the molecules actually occur as such in the protein molecule remains highly doubtful. The method of hydrolysis and separation is often such as to lead to diketopiperazine formation. They are nearly all composed of the simple amino acids. Perhaps the most striking criticism concerning their supposed occurrence in the protein molecule originates from their behaviour toward the proteolytic enzymes.

9. THE ACTION OF PROTEOLYTIC ENZYMES ON DIKETOPIPERAZINES

It will be recalled that the essential criterion for the presence of any configuration occurring among the products of biological origin is the ability of enzymes to attack that configuration. Waldschmidt-Leitz and Schäffner (72) studied the effect of the proteases pepsin, trypsin, and papain, as well as that of the peptidases of erepsin on the neutral glycine anhydride. In all cases their results were completely negative. The more complex diketopiperazines of glutamic and aspartic acids, in which one or more carboxyl groups remain free, were studied by Matsui (73) and Ishiyama (74). It was found that this type of anhydride was apparently very slowly split by activated trypsin (about 10 per cent in 48 hours). Other workers (75, 76) attempted the synthesis of arginine and lysine anhydrides and reported almost quantitative splitting of such molecules with pepsin. The latter results appear highly doubtful (77). The most complex diketopiperazine yet prepared, anhydrol-lysyl-l-glutamide, was completely resistant toward pepsin, trypsin, and papain -HCN (77).20

It appears quite improbable that the diketopiperazines occupy any significant position in the scheme of the proteins. They are useful in the preparative procedure of certain dipeptides (see above) and their chemistry is extremely interesting and suggestive. They

²⁰ Complete resistance to the action of proteases by diketopiperazines with acid side chains has been reported recently by Waldschmidt-Leitz and Gärtner (*Z. physiol. Chem.*, 244, 221 (1936)).

illustrate many of the unique properties of the amino acids.²¹ On the other hand, the evidence in favor of the peptide structure of the proteins has greatly increased in the thirty-five years which have elapsed since Fischer and Fourneau synthesized the first peptide.

The Specificity of the Proteases

In Table IV is listed the specificity of certain peptidases. All of the enzymes described require at least one peptide bond and in addition one free α -NH₂ group or one free α -COOH group, or both. It is clear that the action of this group of peptidases is confined to splitting the terminal peptide linkages of substrates of relatively low molecular weight. None of the true proteases, the enzymes which act upon whole proteins, will split peptides of the type generalized in Table IV. However, Bergmann and his coworkers (85) (86) (87) (88) (89) recently made the striking discovery that the true proteases will split smaller peptides when both the α -amino and α -carboxyl groups at the ends of the molecule are masked by substituent groups. The proteases are therefore also peptidases inasmuch as they split peptide bonds, but the linkages available for them must be in the interior of the protein molecule, far removed from the extremely few α -groups at the ends.

According to Bergmann, the proteolytic enzymes may therefore be conveniently classified into the endopeptidases whose action is confined to central linkages, and the exopeptidases which are restricted to terminal peptide bonds. The enzymic degradation of a protein will therefore begin with the proteases, or endopeptidases, the resulting fragments being subsequently broken down to the amino acids by the exopeptidases. Table IV lists the latter group of enzymes. The former group is listed in Table V.

TABLE V

Enzyme

Crystalline Trypsin
Crystalline Chymotrypsin
Heterotrypsin
Papain
Cathepsin
Bromelin

Substrates

Benzoylarginine amide Carbobenzoxytyrosylglycine amide Benzoylglycyl-lysine amide Benzoylglycine amide Carbobenzoxyglycylglutamylglycine amide Carbobenzoxyglycylglutamylglycine amide

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CHAPTER VII

THE CHEMICAL CONSTITUTION OF THE PROTEINS

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1. THE CLASSIFICATION OF THE PROTEINS

A chemical classification should be based on definite chemical and physical properties of the individual substances; however, such treatment of the proteins is at present impossible. In order to have some scheme by means of which the proteins can be grouped in an orderly fashion, they will be classified in general according to the proposal of the "Committee on Protein Nomenclature."(1)

THE PROTEINS

- (1) Simple Proteins yield on hydrolysis only amino acids or their derivatives.
- (a) Albumins are soluble in water, coagulable by heat, and are usually deficient in glycine.¹
- (b) Globulins are insoluble in water, soluble in strong acids and alkalies, in neutral salts, and usually contain glycine.¹
- (c) Prolamins are soluble in 70-80 per cent ethyl alcohol, yield large amounts of proline and amide nitrogen, and are deficient in lysine. They have been isolated principally from cereal seeds. The alcohol soluble protein from milk is probably not a true prolamin as it contains lysine.
- (d) Glutelins are heterogeneous mixtures of cell proteins obtained by alkaline extraction of the residues after removal of the albumins, globulins, and prolamins.
- (e) Scleroproteins (Albuminoids) are all those fibrous proteins which have a supporting or protective function in the animal organism. In the plant kingdom they are probably represented by cellulose and similar substances.
 - (1) Collagens, the principal supporting proteins of skin, tendons,

¹ The inadequacy of a classification based simply on solubility is clearly shown by the following: One procedure permits over 65 per cent of the total whey proteins to be isolated as "pure" albumin (2) while another method permits 60 per cent of the total whey proteins to be isolated as globulin (3).

and bones are resistant to peptic and tryptic digestion and are converted into an easily digested soluble protein, gelatin, by boiling with water.

A tentative formula for gelatin based on the analysis of its content of glycine (G), proline (P), and hydroxyproline (P) is:

-G.P.X.G.X.X.G.P.X.G.X.X-

- or -G.X.P.G.X.X.G.X.P.G.X.X- where the other amino acids are represented by X.
- (2) Elastins, present in elastic tissues such as tendons and arteries, are digested by trypsin, are not converted into gelatin, and give a negative color test for hydroxyproline (5).
- (3) Keratins are proteins resistant to digestion by pepsin and trypsin, insoluble in dilute acids and alkalies, in water, and in organic solvents, and which on acid hydrolysis, yield such quantities of histidine, lysine, and arginine, that the molecular ratio of these amino acids, respectively, approximate 1:4:12 (6, 8). The cystine content may vary from 0-16 per cent (6).²
- (f) Histones are soluble in water, precipitated by dilute ammonia, and contain large amounts of the basic amino acids, especially lysine and histidine. They are usually found in animal tissues, united as salts, with acidic substances such as heme and nucleic acids.
- (1) Globins are basic proteins (histones) which contain tryptophane, tyrosine, arginine, histidine, and lysine in a molecular ratio of approximately 2:3:3:8:9 (9, 10, 11, 12).
- (g) Protamins are basic polypeptides first found in ripe fish spermatazoa and can be subdivided into four groups depending on their content of the basic amino acids (13); those containing,
 - (1) arginine only (monoprotamins)
 - (2) arginine and lysine (diprotamins)
 - (3) arginine and histidine (diprotamins)
 - (4) arginine, histidine, and lysine (triprotamins)

They usually exist in combination with nucleic acids.

- (2) Conjugated Proteins are proteins united with some substance which on decomposition does not yield amino acids only.
- ² A glycerol extract of the gastrointestinal tract of the larvae of the common clothes moth (*Tineola biselliella* Humm.) contains a proteolytic enzyme capable of digesting keratin (wool) at alkaline reaction in the presence of a strongly reducing substance such as thioglycolic acid (7). The amino and carboxyl groups are liberated in equivalent amounts.

They will be discussed in greater detail under "chemical characteristics of proteins."

The common conjugated proteins are: (a) nucleoproteins; (b) glycoproteins; (c) phosphoproteins; (d) chromoproteins; (e) lipoproteins.

2. METHODS OF ISOLATION

Before giving a detailed description of the chemical characteristics of the proteins, a few general methods for their preparation will be outlined, for it must be remembered that the chemical composition of a protein depends, in part at least, on its mode of preparation (14, 15).

(1) Isolation of Proteins Already in Solution

(a) The preparation of crystalline serum albumin by the "salting out" method.

Serum albumin was one of the first animal proteins to be obtained in crystalline form (16). Horse serum is treated with an equal volume of saturated neutralized ammonium sulfate solution and the globulins are allowed to precipitate at room temperature for 20 hours. The precipitate is removed. The clear filtrate is then carefully acidified with dilute acid to pH 4.6–4.8. After standing at room temperature for some time the crystals are centrifuged off, washed with one-half saturated ammonium sulfate solution and reprecipitated. This is repeated until the crystals are free from amorphous material. A salt-free, preparation can be obtained by dissolving the precipitate and dialyzing (17) at 2° until free from ammonia and sulfate.

(2) Dehydration of Protein Solutions

- (1) Direct: Relatively concentrated protein solutions can be frozen solid and dried in shallow dishes over phosphorus pentoxide in vacuo. The dry powder appears to be quite unaffected by the treatment and can be rendered lipid-free by extraction with anhydrous ether (18) in a Soxhlet apparatus.
- (2). Alcohol: The chilled protein solution is poured into ten volumes of absolute alcohol-ether $(1:1)^3$ at -5° . The precipitate is removed, washed, and extracted with absolute ether.

³ The evidence that proteins are not denatured by brief treatment with cold organic solvents is based upon experiments in which crystalline albumins were prepared from alcohol-ether dried serum. On the other hand, McFarlane (19) has

(3) Acetone: Three volumes of cold acetone are added slowly with stirring to the protein solution. The precipitate is allowed to stand at -5° for 20 minutes, filtered, washed with acetone and ether, and dried in vacuo. The entire operation requires less than 40 minutes.

(3) The Preparation of Albumins and Globulins by the "Salting Out" Method

Chemically and immunologically distinct protein fractions can be obtained from tissue fluids by fractional precipitation with neutral salts. Such protein fractions can be readily obtained by the following method (20):

- (1) If A is the amount of protein solution in cc. to be treated with Z cc. of a saturated salt solution to a/b of saturation, then (A+Z)/Z=b/a or Z=aA/(b-a). The resulting precipitate is then washed with a/b of saturated salt solution and is purified in a manner similar to that described for crystalline serum albumin.
- (2). If a second protein fraction is now to be obtained from the filtrate A^1 already containing a/b=s saturated salt, then Z^1 is the amount of saturated salt solution necessary to obtain a solution a^1/b^1 saturated.

Then

$$\frac{A^{1}+Z^{1}}{A^{1}s+Z^{1}} = \frac{b^{1}}{a^{1}} \quad \text{or} \quad Z^{1} = \frac{A^{1}(b^{1}s-a^{1})}{a^{1}-b^{1}}.$$

A relation between the concentration of salt and the solubility of a protein has been described by Cohn (21) who finds that if s is the solubility of the protein, m is the molecular concentration of the salt, then $\log s = \alpha m + \beta$ when α and β are specific constants. When the log of the solubility in grams per liter of protein is plotted against the molecular concentration of the salt, then α is the slope of the straight line and β is the intercept.

It must always be borne in mind that considerable differences in

observed a shift in the characteristics of the sedimentation diagram of serum treated under these conditions. He believes that treatment in the cold with alcoholether "to be an early stage in the complex phenomenon of denaturation." However, if we think of isolated proteins not as existing in the fluids and tissues of the body as such but as being derived substances artificially produced from tissue proteins by the reagents used in the preparation, (8) then it is evident that identical crystalline proteins can be precipitated by strong neutral salts from protein systems composed of association complexes of different particle size.

the amino acid composition of the protein fractions can be obtained by relatively slight changes in the method used in their preparation. Thus it is of the utmost importance to specify the salt, protein concentration, pH, presence or absence of lipids. temperature, and so forth. In spite of the fact that slight changes in experimental procedure can cause the precipitation of protein fractions differing in their amino acid composition, this must not be understood to mean that identical protein fractions cannot be obtained under different conditions. The ease of preparation of a "homogeneous" protein seems to depend on factors which are still poorly understood. Thus it is quite possible to prepare crystalline edestin (22) of the same amino acid composition by extracting hempseeds, either with 10 per cent sodium chloride at room temperature or with 3 per cent sodium chloride at 50-60°. In contrast to this stable protein, crystalline serum albumin can be divided into several crystalline albumins of different solubilities and amino acid constitutions by fractional crystallization from an ammonium sulfate solution. These fractions can be again combined to yield the original seralbumins (23).

(b) Preparation of albumins and globulins by fractionation with organic solvents.

Aqueous alcohol or acetone can be used to fractionate water soluble protein solutions. Thus it has been claimed that $\frac{1}{2}$ volume of methyl alcohol added to serum completely precipitates the globulins (24, 25). Sumner (26) was able to prepare the first crystalline enzyme, urease, by chilling a 30 per cent alcoholic extract of jack bean meal to -5° .

A detailed comparative study of the amino acid composition of protein fractions obtained by "salting out" and by precipitation with organic solvents should be conducted, for immunological studies indicate no difference between the globulin precipitated by alcohol or by salting out (27).

(4) Isolation of Proteins from Cellular Structures

(a) Extraction. The extraction of protein fractions from tissues and organs is beset with difficulties. An outline of the method of Heidelberger and Kendall (28) will be used to describe the extraction of the cell proteins. Bacteria are suspended in cold acetone and washed by centrifuging (in the cold) with acetone and ether. The dried defatted bacteria are ground in a ball mill until intact cells can no longer be seen microscopically. The material is then ground

with a little 0.2 normal acetate buffer at pH 4 and extracted with a large volume of the same solution in the cold. The residue is removed by centrifugation and extracted with a pH 6.5 buffer. The protein "pH 6.5" is then precipitated with acetic acid at pH 3.8 and removed by centrifugation. The pH 6.5 protein fraction is purified by repeated solution in dilute alkali and reprecipitated with acid. It is finally treated with acetone and ether and dried in vacuo over calcium chloride, sodium hydroxide, and paraffin.

The residue from the pH 6.5 protein fraction is then extracted with dilute ammonia at pH 8 and a second protein isolated. Other protein fractions are subsequently obtained by successive extraction with stronger and stronger alkaline reagents. The subsequent purification of these protein fractions is similar to that described above.

(b) Autoextraction. If sliced frozen pancreas is allowed to thaw, the liquid which drains off contains protein. If the filtrate is now fractionated with neutral salts, a crystalline globulin, trypsin, can be isolated (29).

3. THE CHEMICAL CHARACTERISTICS OF SIMPLE AND CONJUGATED PROTEINS

If we accept the hypothesis that proteins are primarily composed of a series of amino acids united to each other by an acid amide (peptide) linkage, then it is of interest to discuss briefly the influence of the other reactive groups in the polypeptide chain on the chemistry of these substances.

(1) The Free Basic Groups

In previous chapters it has been pointed out that proteins yield three amino acids which have decidedly basic properties. These are histidine, arginine, and lysine, and are characterized by the presence of imidazole, guanidino, and ϵ -amino groups respectively. They are largely responsible for the basic properties of proteins, the quantitative aspects of which are discussed elsewhere in this volume.

(a) Histidine. It is generally considered that the imidazole ring of histidine exists "free" in the protein molecule (30). This conclusion is based on the observation that sturin loses its positive Pauly reaction after treatment with benzenesulfonylchloride (31, 32). However, more recent evidence suggests that, in the case of

certain of the glycoproteins, the polysaccharides may possibly be linked to the peptide chain through the imidazole ring (33, 34).

(b) Arginine. The evidence that the guanidino group of arginine in proteins exists free is based on the following experiments (30): (1) Nitration proceeds in the same way as it does with the amino acid.

$$\begin{array}{l} NH \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \\ | \\ C = NH \\ | \\ NH(NO_2) \end{array}$$

Nitroarginine

(2) The strongly basic clupein, deficient in lysine, has an acid binding capacity equivalent to that of the total amount of "free" arginine obtained after hydrolysis. (3) Unhydrolyzed proteins give a positive Sakaguchi test (see page 188).

It should be borne in mind that the "free" guanidino group can be united in salt or ester linkage to a "free" carboxyl group of one of the acidic amino acids, or with such prosthetic groups as heme, mucoitin sulfuric acid, etc. The interesting compound phosphoarginine (35)

$$\begin{array}{c} \text{NH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CH}(\text{NH}_2) \cdot \text{COOH} \\ \\ \text{C} = \text{NH} \\ \\ \text{NH} \cdot \text{PO(OH)}_2 \\ \\ \text{Phosphoarginine} \end{array}$$

has not been demonstrated in proteins.

(c) Lysine and "Free" Amino Groups. The ε-amino group of lysine apparently does not exist in peptide linkage but influences the reactions of the protein molecule by being "free" or, possibly, united in salt or ester linkage. The evidence for this statement is based on the following: (1) If a lysine containing protein is treated with nitrous acid, no lysine can be isolated after acid hydrolysis. (2) Proteins treated with nitrous acid in the Van Slyke apparatus for 25 minutes yield one-half as much nitrogen as is found by the same method in the lysine fraction after acid hydrolysis of the original protein (36). (3) Proteins containing lysine react with phenylisocyanate (37) to form phenyluramino compounds

while those without lysine (zein) do not. (4) If gelatin, previously benzenesulfonated, is hydrolyzed with formic and sulfuric acids, at least 50 per cent of the original free amino groups can be accounted for by the isolation of ε-aminobenzene-sulfonyl-d-lysine (38).

$$\begin{array}{l} {\rm C_6H_5 \cdot SO_2 \cdot NH \cdot CH_2 \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH} \\ {\epsilon\text{--}Monobenzene sulfonyl-} \textit{d\text{--}lysine} \end{array}$$

In each peptide chain we should expect the presence of one "free" amino group besides those of lysine unless the end amino acid were proline or hydroxyproline. The ratio of the α -amino groups to the total "free" amino groups will depend on the amount of lysine, and on the length of the peptide chain. Thus in zein, no free amino groups have been found (37); in gelatin only 0.5 per cent of the total free amino groups can be assigned to the monoamino acids (38); while the protamin from Sardinia caerulea liberates free amino nitrogen "considerably in excess of one-half of the lysine nitrogen" (39), an observation to be expected in view of its low molecular weight compared to that of the proteins.

If a protein is heated for some time at 150°, the lysine residues are so rearranged (diketopiperazines, internal salts?)

that they become resistant to enzymic hydrolysis (40, 41). In such cases the lysine is not destroyed, for the same amounts can be isolated after hydrolysis of the heated and unheated proteins.

(2) The Acidic Groups

(a) Carboxyl Groups. Proteins are only weakly acidic in spite of the large amounts of the dicarboxylic acids which can be isolated from them. This is because a great majority of these extra carboxyl groups (from aspartic, glutamic, and hydroxyglutamic acids) are present as the acid amides

$$\begin{array}{c|c} \text{RCO-NH-CH-CO-NH-R} \\ & \downarrow \\ \text{CH}_2 \\ \downarrow \\ \text{CONH}_2 \end{array} \xrightarrow{\text{HCl}} \begin{array}{c} \text{HCl} \\ \text{RCOOH+RNH}_2 \cdot \text{HCl} + \\ \text{HOOC} \cdot \text{CH}_2 \cdot \text{COOH+NH}_4 \text{Cl} \end{array}$$

which, on hydrolysis, yields the free amino acid plus ammonia.

In fact it was observed by Osborne, Leavenworth and Brautlecht (42) that the ammonia nitrogen of a great many proteins was equal to the nitrogen of the total dicarboxylic amino acids (NH₃-N:Carboxylic Acid N::1:1). This observation suggested that the "extra" carboxyl group of these substances was not "free" or in peptide linkage but was present as the acid amide. The constancy of this 1:1 ratio in so many different proteins is probably fortuitous when we remember that hydroxyglutamic acid had not yet been discovered. This problem has since been reinvestigated (43). A study of 15 proteins indicated that in six the deviation from the 1:1 ratio was less than 10 per cent, in seven proteins less than 50 per cent, and in two proteins 80 and 140 per cent respectively. Thus we must conclude that the amount of "free" carboxyl groups varies widely among different proteins.

(b) Other Acidic Groups. The phenolic hydroxyl group of tyrosine is probably neutral under the usual physiological conditions but it has base binding capacity in the presence of strong alkali.

(3) Halogens

(A) Iodine. Vogel (44) in 1848 suggested that the iodine found in sponges was present in organic form. This organic iodide was isolated in 1896 (45) and identified by Wheeler and Mendel (46, 47) in 1909. In view of the finding by Baumann (48) of iodine in the thyroid gland, in 1896, it is surprising that iodogorgoic acid (3, 5 diiodotyrosine)

HO
$$CH_2 \cdot CH(NH_2) \cdot COOH$$
3, 5-Diiodotyrosine

was not isolated from the gland until 1929 (49, 50, cf. 51).

The physiologically important iodine containing amino acid is thyroxine, $(\beta$ - [3,5-diiodo-4-(3',5'-diiodo-4'-hydroxy-phenoxy)-phenyl]- α -amino propionic acid.

$$\begin{array}{c} I \\ \hline \\ I \\ \hline \\ Thyroxine \\ \end{array} \\ \begin{array}{c} I \\ CH_2 \cdot CH(NH_2) \cdot COOH \\ \hline \\ \end{array}$$

This compound, first isolated in 1915 (52) and synthesized in 1927 (53) has marked hormonal effects per se. It has been found only in the thyroid gland. This compound is adequately discussed in texts on physiological chemistry.

(b) Bromine. The bromine analogue of diiodotyrosine, 3, 5-di-bromotyrosine, has been found in certain corals (*Primnoa lepodifera*) (54). These are the only known natural sources of a brominated amino acid, although Friedländer (162) obtained 6, 6'-dibromo-indigo from *Murex brandaris* and *P. lapillus*.

(4) Hydroxyl and Phosphorylated Hydroxyl Groups

- (a) Phenolic Hydroxyls. The phenolic groups of tyrosine (diidotyrosine, etc.) have been mentioned above.
- (b) Alcoholic Hydroxyls. Four amino acids have definitely been shown to contain a β -hydroxy group. These are serine, hydroxyproline, β -hydroxyglutamic acid, and β -hydroxy- α -amino-n-butyric acid (55). These hydroxyls exist either "free," esterified with phosphoric acid, or possibly as esters or ethers with other amino acids or such acidic substances as chondroitin sulfuric acid.

(5) Sulfur

(a) Cystine, Cysteine, Djenkolic acid, and Methionine. These four substances are the known sulfur containing amino acids of proteins even though indirect evident points to the possibility of others (56). Methionine can replace cystine of the diet. The chemical behaviour of the methiol group of methionine in the protein molecule awaits investigation. More is known, however, concerning the influence of the cystyl and cysteyl radicals on the structure and function of proteins.

Cystine-containing proteins can be divided into four categories (62, 63). (1) Natural proteins (muscle, thymus histone (64)) which give a positive nitroprusside

 $Na_2Fe(CN)_5NO \cdot 2H_2O$ Sodium Nitroprusside test for –SH groups. (2) Proteins (egg albumin) which give a positive sulphydryl reaction after denaturation. (3) Denatured proteins (serum albumin) which give a positive sulphydryl test after treatment with cyanide. (4) Those proteins which do not give a positive reaction (globin, ovomucoid, brain proteins (65), etc.). Thus we see that in some instances denaturation causes the liberation of previously masked –SH groups, or a reduction of –SS– groups, while in other cases there is an exposing of –SS– groups which after reduction, are capable of reacting with nitroprusside. Vines (66) and Mendel (67) demonstrated that certain proteolytic enzymes could be activated by HCN, H₂S, etc. This reaction is said to be reversible, the inactive enzyme giving a negative nitroprusside test, while the active substance contains –SH (68).

The hormone insulin, on the other hand, contains no sulphydryl groups in the natural active state and is inactive when its disulfide linkages are reduced to -SH by cysteine, H₂S, thioglycolic acid, etc. (69, 70). If the -SH insulin is reoxidized to -SS- by the usual methods, a completely inactive product results. Freudenberg and Wegmann (71) pictured this reaction according to the following model:

 $\begin{array}{l} \operatorname{Ins} \cdot \operatorname{SSR} + \operatorname{H}_2 \rightarrow \operatorname{Ins} \cdot \operatorname{SH} + \operatorname{HSR} + \operatorname{O}_2 \rightarrow \operatorname{Ins} \cdot \operatorname{SS} \cdot \operatorname{Ins} + \operatorname{RSSR} \\ \text{active} & \operatorname{inactive} \end{array}$

They believed that the group—SR is a relatively small molecule and possibly could be replaced by some other physiological sulphydryl compound such as cysteine. This idea was subjected to experimental proof in the following manner. Crystalline insulin was reduced completely with cysteine. The inactive reduced product was then treated with a large excess of cysteine (or glutathione) and carefully oxidized with air or H_2O_2 . The resulting product is active. If thioglycolic acid is used instead of cysteine an inactive product results:

 $\begin{array}{ll} \operatorname{Ins} \cdot \operatorname{SSR} + \operatorname{HSCH_2CHNH_2COOH} \rightarrow & \operatorname{Ins} \cdot \operatorname{SH} + \operatorname{HSR} + \operatorname{Cystine} \\ \operatorname{active} \end{array}$

 $\begin{array}{ccc} \operatorname{Ins} \cdot \operatorname{SH} + \operatorname{HSCH_2CHNH_2COOH} & \operatorname{O_2} & \operatorname{Ins} \cdot \operatorname{S} \cdot \operatorname{SCH_2CHNH_2COOH} + \operatorname{H_2O} \\ \operatorname{inactive} & \to & \operatorname{active} \end{array}$

Attempts to isolate HSR by ultrafiltration, etc., have failed. Thus it is as yet impossible to know whether the cysteine replaces a group of comparable size or not. If the equivalent weight of the

active portion of insulin is 18,000, then the group Insulin S- probably has a molecular weight somewhere between 9,000 and 18,000.

These observations indicate that two peptide chains of dissimilar constitution can be joined through the disulfide group of cystine, that is, each half of a cystine molecule can be part of entirely different peptide chains. They also indicate how a crystalline protein of well defined chemical, physical, and physiological properties can be converted by simple chemical means into another protein of the same highly specific physiological function but which is chemically dissimilar to the original.

Certain keratins contain a large amount of cystine (6). If wool and feathers are reduced with thioglycolic acid, the resulting –SH protein is soluble in acids and alkalies and is digested by pepsin and trypsin (72, 73). On reoxidation of the –SH groups to –SS– by O₂, a new protein is formed which is soluble and digested by enzymes. Thus it seems that the inert, stable, fibrous structure of these keratins is dependent, in part, on the joining together of the peptide chains in a definite manner in space.

The special groupings described so far are those which actually occur within the peptide chain. There are, however, other important groups which are not bound quite so intimately to the peptide chain and which can be separated from it without destroying the protein nature of the polypeptide These special compounds usually do not yield amino acids on hydrolysis and are called prosthetic groups. The chemical characteristics of some of these prosthetic groups and the mode of their union with the proteins will now be described briefly.

(6) Phosphorus

(a) Phosphoproteins. Certain proteins, especially those used for the nourishment of the young, such as casein, vitellin, etc., are not homogenous products as was generally believed but can be fractionated to yield components especially rich in phosphorus (74, 75). Careful hydrolysis of this fraction has shown that the phosphorus is present as the phosphoric acid ester of one of the hydroxyamino acids (76). Lipmann (77, 78) has isolated phosphoserine

 $(HO)_{2}OPO \cdot CH_{2} \cdot CH(NH_{2}) \cdot COOH$ Phosphoserine

from casein and vitellin and claims it to be the only phosphorylated

amino acid in the phosphoprotein molecule.

(b) Nucleoproteins are found in the nucleus of every cell. They are the salts of mono- and tetranucleotides (phosphoric acid-sugarnitrogenous base).4 Osborne and Campbell (80) were the first to demonstrate clearly that nucleoproteins, prepared from the same tissue by slightly different methods, had different ratios of protein to nucleic acid. They showed that such variations could be expected when a polyvalent base (protein) unites in different proportions with a polyvalent acid (nucleic) acid. "In reality 'nucleoprotein' means rather a 'method of preparation' than a chemical substance."

Nucleoproteins are usually prepared by extracting the organ with a dilute alkaline solution (81) The resulting product is either the protein salt of a mononucleotide (e.g., guanylic acid) or nucleic acid (tetranucleotide).

> Nucleic acid (veast) Phosphoric acid-sugar-guanine Phosph.-sugar-cytosine Phosph.-sugar-uracil Phosph.-sugar-adenine (A) Levene

> > Adenine-sugar-phosph.-sugar-uracil phosph. phosph. Cystosine-sugar-phosph.-sugar-guanine (B) Takahashi

The isolation and crystallization of Warburg's yellow "enzyme" is the first instance of the crystallization of a conjugated protein which can be classified as a type of nucleoprotein (82). The vellow "enzyme" has a prosthetic group composed of a substituted pyrimidine (alloxazine), a sugar (ribose), and phosphoric acid. Thus the isolation of the yellow "enzyme" suggests a new series of nucleoprotein-like substances containing flavine (6.7-Dimethyl-9 (d-ribityl)-benzisoalloxazine)

⁴ A nucleoprotein has been obtained from the pancreas which is apparently a pentanucleotide containing three molecules of purine pentose nucleotides and two molecules of pyrimidine nucleotides (79).

instead of simple substituted purines and pyrimidines as the nucleosides. The long-observed occurrence of another pyrimidine derivative, vitamin B₁ (thiamine),

Vitamin B₁ chloride hydrochloride (Williams)

within the cell nucleus suggests that it too might fulfill the function of the nitrogenous base of a "nucleoprotein."

The most interesting nucleoproteins are the chromosomes. It has been observed that the threadlike individual chromosomes are characterized by successions of light and dark bands which are arranged in definite patterns. Wrinch (83) has suggested that these chromosomes are composed of long chains of amino acids joined together by means of their amino and carboxyl groups (see structure). These polypeptide chains, which are laid down in parallel bundles or micelles, contain alternating concentrations of basic and nonbasic amino acids. The basic groups probably arrange themselves on the surface and unite with the nucleic acid molecules. Thus, where there are basic groups in the protein chain, there will be rings of nucleic acid; where the basic rests are lacking, nucleic acid rings will also be lacking. The dark bands of the chromosomes are associated with regions of high density, i.e., nucleic acid rings; the light bands occur where the density is low, in the absence of

nucleic acid. The fundamental units for living matter, the genes, thus appear to be composed of nucleoproteins characterized by a relative concentration of the basic amino acids in certain portions of the peptide chain (contra cf. Bergmann's hypothesis which is given later in this Chapter).

(c) Lipoproteins. If a tissue is extracted with ether until no further soluble substances are obtained and then treated with strong alcohol, it is found that renewed treatment with ether results in the extraction of considerably more material. The protein of egg yolk (lecithovitellin) behaves similarly toward ether and alcohol and in such a way as to leave no other reasonable conclusion that a chemical union, unstable towards alcohol, exists between the protein and phosphatide (85).

Although the lecithoproteins⁵

$$\begin{array}{c} H_2CO(OCR_1) \\ HCO(OCR_2) \\ \hline \\ O \\ H_2C-O-P-O-CH_2 \cdot CH_2 \cdot N \\ OH \end{array} \vdots (CH_3)_3$$

Lecithin (R represents a fatty acid radical)

probably exist in all cells, we have no adequate idea of the mode of linkage between phosphatide and protein. Somewhat similar products have been recently studied by Mâcheboeuf (86, 87) who has prepared a lipoalbumin from serum by acid ammonium sulfate fractionation (pH 3.8 and one-half of saturation). This product forms a perfectly clear solution in water (at pH 7 or above) and contains besides protein, 23 per cent of lecithin and 18 per cent of cholesterol esters. These lipids cannot be extracted by ether until the complex has been treated with hot alcohol. Other experiments by Mâcheboeuf and by Sörensen (23) indicate that the linkage between the lipid and the protein is probably a residual or non-polar valency linkage.

⁵ A crystalline lipoprotein was obtained from cells by simple centrifugation (84). When the crystals were treated with alcohol, the lipoprotein was decomposed, yielding phospholipin and a vitellin.

- (7) Chromoproteins: Iron, Copper, Manganese, Magnesium, Vanadium, Cobalt, etc.
- (a) Iron. Evidence has been accumulating for some time that the conjugated proteins with an iron containing prosthetic group, play the principal part in the respiration of aerobic organisms. As hemoglobin has been the best studied (12, 88, 89) of these compounds, we will confine our discussion principally to this substance. The structure and synthesis of hemin

has been described by Hans Fischer. The amino acid compositions of the globins have been investigated by Vickery (90), Block (12), Roche (9, 10, 11), and others. We may conclude from their studies that the molecular ratios of tryptophane, tyrosine, arginine, histidine, and lysine are approximately as 2:3:3:8:9 for all the mammalian globins thus far examined. On the other hand, the known differences between the mammalian hemoglobins can be accounted for, in part at least, by differences in their sulfur, cystine and methionine content. Thus the molecular ratios of arginine to sulfur to cystine is 75:50:7 for the horse, 75:100:14 for the sheep, and 75:75:21 for the dog.

As the iron content of any hemoglobin is known to be characteristically constant, the fixed ratios of the bases to this element and to one another can be interpreted as supporting the hypothesis that tissue proteins are built upon or around an "anlage" of relatively fixed proportions of arginine, histidine, and lysine (12, 88). "Recent work makes it probable that the linkage between hematin and globin is through an acidic group in the latter and it cannot therefore be argued that a constancy of proportion between iron and a base, like histidine, is a fortuitous result occasioned by the fact that each molecule of hematin is linked to the protein by means of histidine. The proportionality must have a deeper significance" (88).

Recent investigations (cf. 89) have indicated the presence of several hemoglobins in the same individual. Thus it has been shown that the blood of human infants contains two kinds of hemoglobins, the one identical to that of the maternal blood, the other to that of the fetus. It is also possible that there occurs an additional hemoglobin peculiar to pernicious anemia.

The protein components of the iron containing pigments of the invertebrates (erythrocruorins and chlorocruorins) usually yield considerably less histidine than the mammalian globins and are undoubtedly of quite a different amino acid makeup. The chemical composition of the protein moiety of other iron containing respiratory pigments awaits investigation.

(b) Copper (Hemocyanins). Certain invertebrates, such as the octopus and squid, contain a blue respiratory protein in their blood stream. These substances are dissolved directly in the serum and are not, like the mammalian hemoglobins, found in corpuscles. It should be noted that all of the endocellular pigments exist only as relatively small molecules (17,000 to 68,000), while the "plasma" pigments are composed of particles with "molecular weights" of the order of several millions (cf. (89)). The copper content of the prosthetic group of hemocyanins was reported in 1847 (91). At first it was thought that the prosthetic group contained pyrrol rings like those present in hemoglobin but later work indicates it to be a polypeptide which contains copper, considerable sulfur, leucine, tyrosine and probably serine (92). No basic amino acids, tryptophane, cysteine, cystine, or methionine have been found. The hemocyanins show molecular weights in the ultracentrifuge varying from 350,000 to 5,000,000 (93) (see Chapter VIII).

The protein component of Limulus hemocyanin has recently been

analyzed (94) for the basic amino acids by the silver precipitation method (95). Six and one-half per cent of arginine, 4.6 per cent of histidine, and 9.0 per cent of lysine were isolated. As these amino acids are in the ratio of 1:1:2 in comparison to mammalian hemoglobin of 3:8:9, the composition of these two protein types is quite different.

- (c) Manganese. A pigment which contains manganese has been reported (96) in the blood of the worm, *Pinna squamosa*.
- (d) Magnesium (Chlorophyll). Because ether is unable to extract more than a small portion of the chlorophyll

 $\begin{aligned} Phytyl = (CH_3)_2 \colon CH \cdot (CH_2)_3 \cdot CH(CH_3) \cdot (CH_2)_3 \cdot CH(CH_3) \cdot \\ & (CH_2)_3 \cdot C(CH_3) \colon CHCH_2 - \end{aligned}$

from leaves until these have been first treated with alcohol, it has been suggested that this magnesium porphyrin complex is bound to the protein of the cell in a manner similar to the lipoproteins (85).

- (e) Vanadium occurs in considerable quantities in the blood of certain marine animals (ascidians). It probably functions as a respiratory catalyst (166).
- (f) Cobalt and other metals. Evidence is accumulating that certain metals such as Co, Zn, Ni, etc., unite directly with proteins to influence their physiological action. Thus the enzyme arginase is seemingly inactive without the presence of some Co++, Ni++, Mn++, or Fe++. These metals also react with crystalline urease to render this supposedly specific enzyme capable of hydrolyzing arginine to ornithine (97).

Scott (98) has reported that insulin cannot be crystallized unless a certain minimal proportion of a metal such as Zn (0.52 per cent), Cd (0.77 per cent), or Co (0.44 per cent) is present. These amounts are equivalent to 1.6, 1.4, and 1.5 formula weights of the metals (for a molecular weight of insulin of 20,000) thus suggesting that these metals are in chemical combination with insulin. However, no relation was found between the zinc content of fresh pancreas and the amount of insulin which could be obtained.

(8) Glycoproteins: Sugars, Polysaccharides, Polyuronic Acids, etc.6

(a) Glucidoproteins. Fränkel and Jellinek (99) obtained a nitrogen containing polysaccharide from crystalline egg albumin. The polysaccharide is probably a polymerized glucosamine-mannose (100)

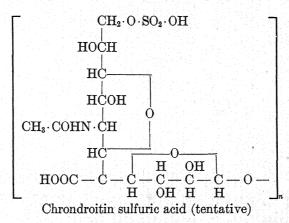
Glucosamine-mannose (M represents mannose)

apparently united to the peptide chain through the imidazole ring (33). At present it is undecided if the carbohydrate portion of these protein complexes originates from contaminating mucoid or whether it is actually united to the protein moiety in the form of a reversibly dissociable component system. Examples: crystalline ovalbumin, seralbumin, etc.

- (b) Mucoids do not contain a uronic acid but consist of a polypeptide firmly united to a polysaccharide such as polymerized glucosamine-mannose. The polypeptide is usually rich in organic sulfur but no inorganic sulfate is present in this group (101, 102). Examples: serum mucoid, ovomucoid.
 - (c) Mucins are characterized by containing a uronic acid.

⁶ This classification is based on unpublished experiments of Dr. Karl Meyer. I am greatly indebted to him for permitting me to use these data.

- (1) True mucins do not contain sulfuric acid.
- (a) The carboxyl group of the uronic acid is probably united in salt linkage to the basic groups of proteins. Examples: vitreous humor, Wharton's jelly of the umbilical cord.
- (2) Sulfomucins. These contain sulfuric acid and uronic acid and are probably united to the basic groups of the proteins (cf. Meyer, K., Palmer, J. W., and Smyth, E. M., J. Biol. Chem., 119, 501 (1937)).
- (a) Chondrosulfomucins are derived from mesodermal tissue and consist of a protein or polypeptide united with chondroitin sulfuric acid (the diglucoside of glycuronic acid N-acetyl-chondrosamine) (103).



Examples: cartilage, intestinal tissue, etc.

(b) Glucosulfomucins differ only from the chondrosulfomucins in that they yield glucosamine (103) in place of chondrosamine. Examples: cornea, gastric mucosa.

4. INTRODUCTION OF SPECIAL GROUPS INTO THE PROTEIN MOLECULE

In the chemical investigation of proteins it is often desirable to earmark particular groups. Such methods permit the identification of the marked amino acids after hydrolysis. Some of the methods used to accomplish this are:

(1) Methylation and Esterification. The methyl group of methionine is the only known methyl group present in proteins which can be split by hydrogen iodide (104). However it is possible to methylate (esterify) proteins (64, 105) by suspending the finely

powdered material in anhydrous methyl alcohol and treating the suspension with dry hydrogen chloride. The protein methyl ester hydrochloride is produced. This permits the calculation of the number of carboxyl and amino groups present, the former by a methoxy, the latter by a chloride determination. It is of the utmost importance that anhydrous conditions be maintained in order to prevent the protein from hydrolyzing. Protein methyl ester can be further methylated by treatment with dimethyl sulfate at pH 8 to 9 or by diazomethane. The resulting products no longer give a positive Pauly diazo test for the free imidazole ring. It is also probable that methyl groups are introduced into the hydroxyl group of tyrosine (absence of the Millon reaction) and the beta hydroxyl group of the amino acids.

The proteolytic enzymes of the intestinal tract do not split methylated proteins (107).

(2) Acylation. Felix and his associates (108, 109) have completely benzoylated clupein (free from histidine or lysine) with benzoyl chloride so that each hydroxy and guanidino group reacts with one molecule of benzoyl chloride. An arginine determination permits the estimation, by difference, of the hydroxyl groups. If benzoyl clupein is acetylated, one molecule of acetic acid unites with each benzoylated guanidino group. Acid hydrolysis of the acetylbenzoyl clupein permits the isolation of all but one of the original arginine molecules. The remaining molecule has been converted into ornithine which indicates its presence at one end of the peptide chain.

Another acylating reagent, benzenesulfonylchloride, reacts with gelatin at pH 10 to 11. Under these conditions no measurable hydrolysis occurs. The resulting product, after hydrolysis, yields ←benzenesulfonyl-d-lysine.

Benzenesulfonated gelatin is not digested by pepsin and is somewhat less rapidly and completely hydrolyzed by trypsin (38) than untreated gelatin.

(3) Nitroproteins. (Xanthroproteins.) The treatment of proteins with concentrated nitric acid results in a yellow product (xanthoproteic reaction) due to the simultaneous nitration and oxidation. The colored products are presumably the result of nitra-

 $^{^{7}}$ It is said that under these conditions there are no alterations in the protein except the conversion of $-S \cdot S - to - SH$. However Kiesel (106) claims that esterification of glycinin causes destructive and hydrolytic changes in the protein molecule.

tion of the benzene rings of tyrosine and tryptophane. Nitrated proteins give negative Millon and alkaline lead reactions (107).

(4) Halogenated proteins. In addition to the naturally occurring iodine and bromine containing proteins, iodine can be introduced into the protein molecule (I₂+alkali). Hydrolysis of such iodized proteins yields diiodotyrosine. However, at present, no physiologically active iodized protein has been prepared. Under more strenuous conditions, halogen can be introduced into the imidazole and indole rings. Iodized proteins have a markedly acidic character permitting solution by alkali and precipitation by acid (107).

Treatment of proteins with bromine in alkaline solution causes destructive oxidation of the cystine (56).

5. HYPOTHESES OF THE STRUCTURE OF PROTEINS

The general constitutional features of the fats and carbohydrates are relatively simple to understand, whereas the proteins are much more complex. Substances which can be classified as proteins have been obtained with molecular weights varying from a thousand to 300 million. Although such substances show a wide range of physical, chemical, and physiological properties, all our evidence points to the fact that they are constructed along essentially similar lines. Proteins consist of chains of amino acids which are bound to each other through their amino and carboxyl groups. The stability of the naturally occurring proteins is not a constant characteristic. Probably the most stable protein in the body is keratin which can be subjected to various strenuous physical and chemical manipulations. Keratin is essentially a "dead" or partly denatured protein, produced by the organism from living precursors. Contrast this to the behaviour of a living protoplasmic system such as blood plasma, which is in so delicate a state of equilibrium that mere contact with an inert foreign body causes the complicated process of coagulation. Between these two extremes there are proteins and protein systems of all degrees of stability. These facts must constantly be borne in mind in all protein studies and in all deductions based on such studies. If the properties of a stable protein like keratin are investigated, the dead isolated material will behave as it did in the natural living state. But if the protein came from a metabolically active cell or tissue the properties of the dead protein are other than those exhibited by it as a living unit of the organism.

In this section an attempt will be made to discuss the principal hypotheses concerning the structure of the protein molecule. Ideas, well received at the time of their publication but now generally modified or supplanted by newer and more suitable hypotheses, will only be mentioned briefly. For an excellent review of the earlier speculations on protein structure, the reader is referred to that of Vickery and Osborne (110, 111).

(1) The Protamin Nucleus Hypothesis (Kossel and Siegfried)

On returning from the sea to fresh water, salmon do not consume any food. In their journey up the rivers large amounts of muscle protein must be broken down in order to supply energy for locomotion and materials to build the rapidly hypertrophying gonads which increase from 0.1 per cent to about 6 per cent of the total body weight (112). Because the arginine content of these organs is very much higher than in the muscle, Kossel (113) has suggested that the muscle protein is divided during catabolism into parts, the greater used for energy, the smaller, rich in arginine, used for the formation of the gonads. He called this process "biological reduction" and pictured it somewhat as follows: muscle protein—histone—triprotamin (arginine, histidine, lysine)—diprotamin $\binom{(A, H)}{(A, L)}$ —monoprotamin (A).

The highly basic protamins, which yield up to 87 per cent of their total weight as arginine, can be isolated from ripe fish sperm (13). This observation, coupled with the fact that arginine is apparently the one amino acid present in all proteins, led Kossel (114, 115) to the view that proteins contain a central basic nucleus rich in arginine around which the albuminoses and peptones, (now called polypeptides), relatively deficient in the bases, are joined in a weak, easily broken linkage.

The most studied of the protamins is the monoprotamin, clupein, the constitution of which is still subject to controversy. Thus Felix (108) has claimed that it is a mixture made up of the following components:

Clupein A₁ (4 arginine, 1 serine, 1 alanine)

Clupein A₂ (4 arginine, 1 proline, 1 amino valeric acid)

Clupein B $(A_1+A_2-H_2O)$

Clupein C $(B+B-H_2O)$

While Waldschmidt-Leitz (116) has given the formula for an unfractionated clupein prepared by a slightly different method as:

M-A-A-M-A-A-M-A-A-P-A-A-M-A-A

where M represents monoaminomonocarboxylic amino acids, A represents arginine, and P stands for proline.

On account of the very low amount of the arginine in some proteins, and in the light of other studies, the protamin nucleus hypothesis as originally proposed by Kossel (114, 115) has not been generally accepted. Proteins are probably not built upon a central basic polypeptide nucleus but are composed of numerous polypeptide chains, some rich in basic, some in acidic, and some in neutral amino acids. However, an "anlage" of the basic amino acids seems to exist which is characteristic for any one biological type of protein (cf. (8)).

(2) The Peptide Theory (Hofmeister and Fischer): Proteins consist of chains of amino acids bound to each other through their amino and carboxyl groups (peptide linkage)

$$(NH_2)CH \cdot COOH + (NH_2)CH \cdot COOH \rightarrow R \cdot CH \cdot CONH \cdot CH \cdot COOH$$

$$R \qquad R \qquad NH_2 \qquad R$$
or
$$(NH_2)CH \cdot COOH + HN - CH_2 \qquad (NH_2)CH \cdot CON - CH_2$$

$$CH_2 \rightarrow \qquad CH_2 \rightarrow \qquad CH_2$$

$$R \qquad CH - CH_2 \qquad R \qquad CH - CH_2$$

$$COOH \qquad COOH$$
The Peptide Bond

The adequacy of an hypothesis is measured by its ability to fit the experimental facts. The first successful theory of the structure of the protein molecule was arrived at almost simultaneously by the physiological chemist, Hofmeister (117) and the organic chemist, Fischer (118). Even before 1900 it was known that when proteins are hydrolyzed by the aid of acids, alkalies, or enzymes the principal end products are amino acids. Therefore in order properly to postulate the composition of the proteins, it was necessary to picture the various ways in which amino acids might be linked to each other. Hofmeister (117) discussed these linkages as follows. A direct union via carbon atoms was eliminated as, in this case, the decomposition of proteins by acids, alkalies, and especially by enzymes was impossible to explain. The union of amino acids by means of an ether, ester, or anhydride linkage was also dismissed for if this combination occurred to any considerable extent, hy-

droxyamino acids should be much more common protein degradation products than they have been found to be. Furthermore, as this type of combination leaves the amino groups exposed, we would expect a substance so constituted to be strongly basic in character. The more probable mode of combination was therefore through a nitrogen atom. Three possibilities were suggested:

The first linkage occurs in proline and hydroxyproline and is stable. The second type is found only in one amino acid, arginine, and is also stable to acid hydrolysis. The evidence that the third mode of combination, called the peptide linkage by Fischer, is the one involved in uniting the amino acids of proteins was clearly summarized by Hofmeister. This evidence when brought up to date is as follows:

- (1) Native protein itself contains very little amino nitrogen but the end products of protein hydrolysis contain large amounts.
 - (2) The biuret reaction

$NH_2 \cdot CONH \cdot CO(NH_2)$ Biuret

is given by many substances which contain the peptide bond.

- (3) Synthetic peptides have been prepared which can be digested by enzymes.
- (4) The peptide union is encountered in nature, i.e., hippuric acid,

C₆H₅·CONH·CH₂·COOH Hippuric Acid

(5) Hydrolysis of proteins, either by acids or enzymes, causes the liberation of amino and carboxyl groups in equivalent amounts.

The peptide hypothesis has been the only theory on the structure of the protein molecule which has thoroughly withstood the test of time and experimentation, and upon it are based the only protein theories which have obtained acceptance during the past 25 years.

However, the peptide theory as originally proposed does not adequately explain certain facts, among which are the high molecular weight and denaturation of the proteins and the insolubility of the keratins. In attempting to fit these facts into the theory the following hypotheses have been proposed.

(3) The Diketopiperazine Hypothesis (Abderhalden)

Amino acid anhydrides (diketopiperazines)

can be obtained from proteins after partial hydrolysis. However, these compounds can readily be formed by the condensation of two amino acids or by the cyclyzing of a dipeptide. Furthermore it appears that diketopiperazines are not split by the usual proteolytic enzymes (cf. (119)). Nevertheless, Abderhalden (119a) suggested that proteins are composed of a number of diketopiperazine-containing complexes which are held together by means of secondary valences. These elementary "complexes," to use Abderhalden's somewhat paradoxical phrase, "may differ in number, constitution, and arrangement in different proteins, but, since these are held together only by secondary valences, it is possible to conceive that, by changes in arrangement and perhaps the loss of a few of the complexes, alterations may take place that could even go as far as to convert one protein into another" (110). Abderhalden suggested that the action of pepsin was the breaking up of these secondary valences with the liberation of the diketopiperazine complexes. Further hydrolysis opened the diketopiperazine ring structure and finally the peptide bonds were split. This theory more or less plausibly accounts for many of the peculiar properties of proteins but unfortunately the evidence upon which it rested was very meagre at the time it was first proposed. In an effort to find diketopiperazine structures in proteins, Abderhalden carried out many experiments but he failed to establish either by actual isolation or by colorimetric tests that true diketopiperazine rings ever exist in natural undenatured proteins. Since all the experimental evidence

upon which this idea rested has been disproved the diketopiperazine hypothesis, as originally formulated by Abderhalden, should no longer be given any credence, although it is possible that some proteins do contain a number of diketopiperazine rings (see Chapter VI).

Since the above was written, Waldschmidt-Leitz and Gärtner (119b) have reported that glycylglutamic acid anhydride and aspartic acid anhydride are not hydrolyzed by purified or crude pancreatic or ereptic enzymes or papain. They believe that the so-called hydrolysis of such diketopiperazines as have been reported must, therefore, have been based upon an experimental error. This investigation in conjunction with those on the action of enzymes upon synthetic and natural substrates (cf. 128, 129, 130, and others) indicates that the search for specific structures (e.g., diketopiperazine rings) in proteins to explain enzymic specificity is now superfluous.

(4) The Periodicity of Amino Acids in Peptide Chains (Bergmann)

The observations of Kossel (113) and others (116) that amino acids tend to repeat themselves periodically in the protein chain have been extended by Bergmann (120) and Bergmann and Niemann (121, 122). These investigators observed that the numbers of molecules of the amino acids (which are present in the higher proportions) appear to be in a simple arithmetical ratio. "This relationship cannot be fortuitous and must represent a regularity of the structure of the protein itself." These investigators propose the following hypothesis: "In every protein each amino acid residue is distributed throughout the entire peptide chain at constant intervals, i.e., each amino acid residue recurs with a characteristic whole number frequency." Some of the data on which this idea is based can be summarized thus: hemoglobin yields on hydrolysis amino acids whose average weighted molecular weight is approximately 133.5. This gives an average amino acid residue weight of 115.5. From this value, it is calculated that every 100 gm. of globin must contain about 0.865 gm. molecule of an average amino acid residue. It was found that globin yielded lysine 8.0, histidine 7.4, aspartic acid 6.4, glutamic acid 3.5, tyrosine 3.3, proline 2.1, arginine 3.1, and cysteine 0.5 per cent. From these results Bergmann and Niemann calculated that the above amino acids comprise 1/16, 1/18, 1/36, 1/48, 1/48, 1/48 and 1/192, respectively, of all the constituent amino acids. In other words, if we could look along a globin polypeptide chain, we should expect to see every lysine residue separated by 15 other amino acid residues, every histidine residue by 17 other residues, every cysteine by 191 others, etc.

The frequency 16, 18, 36, 48 and 192 also indicated that globin must contain 576 amino acid residues or a whole number multiple thereof. When this number, 576, is multiplied by the molecular weight of the average residue, 115.5, we come to 66,500, which is remarkably close to the molecular weight of hemoglobin determined by purely physical methods.

From similar calculations, Bergmann and Niemann find that crystalline egg albumin contains 288 amino acid residues of average molecular weight 124. The product of these two values gives egg albumin a minimum molecular weight of 35,700, which is in good agreement with Svedberg's value of 34,500 determined with the aid of the ultracentrifuge.

These and other data suggest that proteins are built up of 288 amino acid residues or some simple multiple thereof. Thus the Svedberg unit of molecular weight, approximately 34,500, contains 288 amino acid residues, that of molecular weight approximately 69,000 contains 576 residues, that of 108,000 should contain 864, etc. It also follows that the Svedberg unit is not an absolute value dependent on *molecular weight* but it is a variable, subject to the average residual weight of the constituent amino acids.

Protein molecules, therefore, contain a great number of minor superimposed frequencies depending upon the concentration of the individual amino acids, and one or more major frequencies $(n \times 288)$ which are a consequence of some, as yet unknown, law governing their structure. It should be remembered that although similar combinations of a few amino acid residues may occur at certain intervals along the peptide chain, in every case each combination will lie in a different environment. Therefore, no two units in any peptide chain possess identical structural significance.

This type of investigation should enable us eventually to determine accurately the number of amino acids in an individual peptide chain, the exact order in which these amino acids are attached, and to gain an insight of the structural formula of a Svedberg unit. Bergmann's "periodicity hypothesis" is especially applicable to the long-chain fibrous proteins such as silk fibroin (288×9 residues or molecular weight 217,000), wool (288×2 residues or 68,000 molecular weight), and the protein of the neurofibrils.

(5) The Pyrrole Hypothesis (Troensegaard)

In 1921 a paper was published by Troensegaard (123) in which it was suggested that proteins are not composed of amino acids in peptide linkage but are united in the form of condensed pyrrole rings which yield amino acids on hydrolysis.

This theory was based on the following observations: (1) Proteins give a "pine splinter" test for pyrrole. (2) Acetylated proteins, reduced with sodium and amyl alcohol, yield various nitrogen containing compounds on hydrolysis. The pyrrole hypothesis was criticized by Vickery and Osborne (110) and was refuted by experiments of Wrede (124) who showed that similar products

 $NH_2CH_2 \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH + CH_3 \cdot COOH + CH_3 \cdot CO(Cl)$

Reversible Conversion of Lysine into d, l- β -Acetylamino α -homopiperidone (I)

could be obtained by treatment of amino acids under the same conditions that had been used for their supposed isolation from proteins.

(6) Racemization of Proteins and Polypeptides (Dakin and Levene)

Kossel and Weiss (125) were the first to show that when proteins were digested at low temperatures with dilute alkali, there was a rapid decline in optical activity, but in no case was it completely abolished. When the racemized protein was hydrolyzed, inactive histidine, arginine, ornithine, and some inactive lysine were obtained. This finding was extended by Dakin (126, 127) who suggested that the peptide linkages were enolized by alkali.

$$CH-CO-NH- \rightleftharpoons -C = C-NH OH$$

Enolization of Peptide Bonds by Alkali

On acidification of the enolized product and subsequent hydrolysis, the amino acids, whose optical activity is due to the α -carbon atom, were no longer active. These racemized proteins showed absolute stability against the common proteolytic enzymes. This suggests that under the conditions employed (treatment with 0.5 normal sodium hydroxide at 37° for 18 to 20 days) practically all the peptide bonds must have been enolized. Otherwise, if a few of the optically active groups retained their natural configuration one would have expected enzymic hydrolysis at such points.⁸

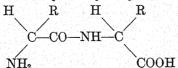
⁸ The fundamental importance of stereochemical arrangements of the amino acids in the structure of proteins has recently been most clearly demonstrated by Bergmann and his associates. Their experiments have shown the requirements necessary for splitting peptide bonds by dipeptidase (128), carboxypeptidase (129), aminopeptidase (129), crystalline trypsin (130), and other enzymes. In this discussion only hydrolysis by the enzyme dipeptidase will be described in some detail.

Dipeptidase splits every dipeptide which has the constitution given in (a).

Stereochemical Aspects of Dipeptidase Action

(a) A dipeptide capable of hydrolysis by dipeptidase.

The requirements for enzymic hydrolysis are:



1. A free amino group separated from the peptide linkage by one carbon atom.

2. A free carboxyl group also separated from the peptide linkage by one carbon atom.

Analogous investigations were carried out more recently by Levene and his collaborators (132, 133) who employed synthetic polypeptides and diketopiperazines. He concludes, "that migration, under the influence of alkali, of the hydrogen atoms attached to asymmetric carbon atoms takes place only on the central amino

- 3. The peptide linkage must contain a hydrogen atom on the nitrogen.
- 4. The α and α -carbon atoms must have at least one hydrogen atom each. If even a single one of these conditions is not fulfilled, enzymic action does not take place.

The enzyme must combine simultaneously with the peptide linkage, the amino and the carboxyl groups in order to hydrolyse the substrate and in doing so, it forces the dipeptide into a very definite spatial arrangement transforming the peptide linkage into its enol (imino) form. If this triple contact is prevented by the blocking of any of the chemically reactive groups or by steric hindrance, enzymic action does not take place. Similarly, Bergmann has shown that other enzymes can act only when other, equally rigorous, conditions are fulfilled by the substrate. Out of these enzyme experiments a new method was developed for the isolation of the individual amino acids. This method is based on the formation of insoluble complex metal salt compounds (4, 131). The steric configuration as well as the chemical properties of the complex metal salt is chosen so that it, like an enzyme, will react in a highly specific manner with the desired amino acid.

(b) Enzyme is effective.

Both H-groups are in front of and both R-groups behind the plane of the paper.

(c) Enzyme is ineffective due to the amino and carboxyl groups being far apart or to hindrance of the approach of the enzyme on account of replacement of one α -hydrogen.

acids of a polypeptide chain, the terminal amino acids which carry the free amino or the carboxyl group remaining wholly unaltered. The prediction of Dakin that racemization in polypeptides would take place only in systems composed of at least three amino acids was thus substantiated." These quantitative studies on the rate and degree of racemization of polypeptides led to the following further conclusions: (1) The degree of racemization of polypeptides is dependent upon the structure of the amino acids; (2) In polypeptides larger than tripeptides, the degree of racemization of the racemizable amino acids seems to be independent of the number of constituent amino acids; (3) The progress of racemization of proteins is consistent with the theory of the polypeptide structure and not with the theory of diketopiperazine structure.

(7) Proteins as Reversibly Dissociable Component Systems (Sörensen)

In 1908, W. B. Hardy (134) observed that untreated serum is readily filtered through a "porous pot." However if the serum was treated by diluting with water, acidification, addition of neutral salts, etc., a fraction was produced, the molecular weight of which was so great that the molecules were arrested by the same filter. Furthermore he showed that the serum protein is electrically inactive. "Neither the whole nor any fraction moves in a field. It is impossible to detect a trace of 'ionic' proteid. Dialysis or dilution disturbs the equilibrium and 'ionic' globulin appears and can be swept out of the general mass of proteid as an opalescent cloud." On the basis of such studies he concluded that "on the whole the balance of probability is against it (that serum is a mixture of certain proteins in solution) and in favor of their being in serum some (possibly one) complex proteid which breaks down readily into fractions whose composition and properties depend upon the degree of dilution and the reagents used."

Hardy's investigations indicated that some, at least, of the soluble serum proteins are artificial products produced by the reagents employed in their preparation. This aspect of Hardy's work was largely neglected until the extensive investigations of S. P. L. Sörensen (23) and his collaborators. A summary of Sörensen's investigations was published in 1930 and deserves thorough study by all interested in the chemical constitution of the proteins. Following on the work of Hardy and of Chick (135, 136), Sörensen observed that the solubility of some purified proteins in dilute salt

solutions varied with the amount of the solid phase. As this was in disagreement with Gibbs' law, it indicated that this crystalline protein must either be composed of a simple mixture or of easily dissociable components which have varying degrees of solubility. By proper fractionation, it was possible to obtain from many times recrystallized serum albumin, various crystalline serum albumins of differing physical and chemical (amino acid) composition which, when combined in the proper portions, again yielded a crystalline protein having the properties and composition of the original. Analogous results have also been obtained with gliadin (137), casein (138), and other proteins.

Thus it appears that certain proteins which have previously been regarded as single substances (chemical individuals) must now be looked upon as mixtures of two or more components. It seems that these proteins do not exist as a simple mixture, but as a system of reversible components so combined that the system behaves osmotically as a single substance. This reversible combination permits an interchange between the components of the system when changes in the state or composition of the liquid phase (temperature, salt content, hydrogen ion activity, etc.) take place. If the opportunity for such a rearrangement of components is presented, the more insoluble co-precipitation system formed under the new conditions will precipitate. Sörensen (23) summarizes his studies in the following manner: "Our investigations cover only soluble proteins, i.e., proteins which may be dissolved in water or alcohol with or without the presence of salts, at neutral, acid, or alkaline reaction, without suffering irreversible decompositions. These substances are represented by the ordinary formula $A_x B_y C_z \dots$, where A, B, and C etc., indicate entire complexes, namely polypeptides, whereas the subjoined indices x, y, z, etc., indicate the numbers of the said complexes contained in the whole component system. Within each complex all the atoms or atom groups are interlinked by main valences whereas the various complexes or components are reversibly interlinked by means of residual valences." Thus it is apparent that if a certain component is especially rich in one amino acid, appropriate physicochemical treatment (salting out, electrodialysis, etc.) would cause a rearrangement of the co-precipitation systems permitting the isolation of a "protein" especially high in this particular amino acid. Thus it has been

⁹ The term dissociate is used in the sense employed by Sörensen.

shown that orosin¹⁰ can be fractionated by neutral salts to give heat coagulable proteins yielding on acid hydrolysis from as little as 4 per cent to as much as 39 per cent of lysine (8, 19). Other investigators have obtained analogous results.¹¹ Such chemical and physical investigations offer strong evidence that most if not all of the soluble, labile proteins found in actively metabolizing protoplasm do not exist as definite chemical individuals (8), i.e., in the form in which they can be isolated, but are probably united by loose secondary linkages with the other polypeptides present, as well as with the lipids (86) and possibly the carbohydrates (142).

The ease with which an isolated protein dissociates9 varies markedly. Thus crystalline hemoglobin dissociates little if at all; egg albumin only very slightly, while serum albumin, serum globulin, casein, gliadin, etc. can be broken up into co-precipitation systems differing considerably in their physical and chemical composition. Soluble proteins, which completely obey the laws of solution and give no indication of dissociability, probably consist of components which are chemically identical compounds. Examples are: the crystalline proteins, trypsin (29), pepsin (143), and ovalbumin which appear to be true chemical individuals. Trypsin is prepared by removing the associated tissue proteins by the use of acidified neutral salts and it is probable that in the cell the enzyme exists united to the other tissue proteins in a reversibly dissociable system. Sörensen suggested such a concentration of individual components when he said "the component in question . . . would probably combine with components of the same kind to form a component system . . . containing only the amino acids present in the involved component."

In further support of Sörensen's hypothesis mention can be made of a purely chemical study of cattle orosin (14). It was observed that cattle serum could be fractionated by various neutral salts to yield albumins and globulins of differing basic amino acid composition. Furthermore, the molecular ratio of lysine to arginine increased with the increasing solubility of the protein in neutral salt

¹⁰ Orosin, from the Greek, ὀρόs or ὀρρόs (serum) is used to designate the total coagulable protein of the serum.

¹¹ Protein fractions yielding approximately 6.6 per cent of histidine (139) or 25 per cent of lysine (140) respectively have been obtained from mammalian orosin. The degree of segregation of the components (polypeptide chains) rich in these amino acids is evident when one remembers that mammalian orosin itself yields only about 2 per cent of histidine and 7 per cent of lysine (141).

solution. These studies lend weight to the concept that serum (and by analogy other active biological systems) does not contain several independent proteins, and that the protein fractions, isolated by physico-chemical methods, are not pre-existent in the serum but are produced by the technique employed in their preparation (15, 141). In these experiments, analytical evidence is presented demonstrating that the proteins which could be isolated by simple chemical treatment with neutral salts do not pre-exist as definite chemical entities in serum, but rather that serum protein is composed of easily associable and dissociable components. Chemical analysis of these co-precipitation systems affords us some insight into their structure: the arginine content of all the protein fractions from cattle serum remained relatively constant at about 5.7 per cent, while the lysine varied from 4.3 per cent in the most insoluble fraction to 39.6 per cent in the most soluble one (14). Thus it must be concluded that the basic amino acid composition of isolated serum albumins and globulins depends, in a measure at least, on the mode of preparation of the fractions and that the lysine to arginine ratio of albumin is always higher than that of globulin.

Experiments such as those described above throw strong doubt on the fundamental validity of attempting to classify the dissociable proteins as albumins, globulins, prolamins, etc. The experiments indicate that the soluble dissociable proteins do not exist in the tissues and organs as such but are produced by the reagents employed in their preparation. Furthermore it is these very classes of proteins which have failed to be characterized by their chemical (amino acid) composition. In contrast, the stable, firmly associated proteins (hemoglobins, keratins) have been so classified.

(8) Directive Influence of the Basic Amino Acids on the Structure of Proteins

The Basic Amino Acid "Anlage" Hypothesis (Block). A measure of success of a classification of proteins founded on amino acid composition was first attained with the keratinoid tissues (6) and the protamins (13). The protamins, however, on account of their relatively simple amino acid composition, seem to form a special class of proteins. This left the keratins as the only true proteins which could be classified on a purely chemical basis (see above). It was shown that even though the actual percentage composition

of the basic amino acids might vary widely, the molecular ratios of histidine, lysine, and arginine, remained approximately constant at about 1:4:12. Further studies of the basic amino acid composition of tissues (141, 144) (orosins) or of the more stable proteins (12) (hemoglobin) indicated that these substances too were capable of such a characterization. Thus the molecular ratio of the basic amino acids yielded by various proteins and tissues (mammalian orosins, avian orosins, mammalian liver proteins, the proteins of entire animals, yeast proteins (145) and forage grass proteins (146) was approximately constant for each group studied. The conception of the "orosin molecule" must not be understood to imply that analogous tissue proteins have a fixed amino acid composition. The molecular ratios of the basic amino acids are constant but the order of the basic amino acids in the peptide chain may vary among homologous tissue proteins. This constancy is of special interest because the content of other amino acids can vary. For example, the molecular ratio of histidine: lysine: arginine in finger nails from normal and from arthritic persons, remains constant (147). However, the cystine content of the normal finger nails is approximately 12.0 per cent while arthritic finger nails contain only about 9.6 per cent of this amino acid. This is a significant difference! Thus a chemical characterization of proteins can be achieved only by analyzing the tissue proteins. At the present time it appears that the basic amino acids, arginine, histidine, lysine, have a directive influence on the general structure and properties of the tissue proteins, although other amino acids especially the dicarboxylic acids may serve in a similar manner. As a result of such experiments, it has been proposed that the tissue proteins can be classified by their content of arginine, histidine, and lysine. The structure of these proteins is dependent upon the relative proportions of the basic amino acids therein which thus function as a directive influence or anlage¹² on the tissue proteins. This suggestion differs from the earlier proposals (Kossel) which postulated that the characteristic proteins, isolated from natural sources, contain a central protamin nucleus (114, 115). Block's view stresses the primary importance of the basic amino acids in the genetic and embryological development of the tissue proteins as they exist

¹² Anlage is employed in the usual biological sense, i.e., as a determiner. Definition—"The embryonic area in which traces of any part first appear; the first aggregation of cells which will form any distinct part or organ of the embryo (148)."

in protoplasm (see page 291 for the importance of the basic amino acids in the structure of chromosomes). This theory accounts for the known variations among analogous proteins obtained from different species of animals as well as for their general similarities.

(9) Molecular Weights of Proteins (Svedberg)

Although a more detailed discussion of the determination of the molecular weight of the soluble proteins will be given elsewhere in this volume (see Chapter VIII) a brief mention should be made of the highly important experiments of Svedberg and his collaborators (93, 149, 150, 151, 152) as they pertain to the structure of the soluble proteins. Svedberg has developed a centrifuge capable of such rapid rotation that soluble molecules of varying sizes can be separated from one another. Thus, one is able to use this ultracentrifuge to determine the molecular weight or particle size of many substances in solution. Svedberg found that the molecular weight of the homogeneous native proteins are apparently simple multiples or sub-multiples of 34,500, which is the molecular weight of ovalbumin. Therefore only a very limited number of molecular weights are represented among the proteins, even though these differ widely with regard to chemical composition. This means that chemically different proteins may have the same molecular weight. In fact, the lowest molecular weight for a real protein (protamins are not generally considered real proteins) is approximately 17,000, while hemocyanin from the snail, Helix pomatia, even in untreated blood, gave a molecular weight of above 5,000,000. Nevertheless only a dozen graded steps are required to proceed from the lowest to the highest molecular weight (particle size).

Svedberg has also found that these soluble proteins are often capable of reversible association and dissociation into molecules whose molecular weights are usually some simple multiple or submultiple of the original (34,500), an observation which supports the idea that proteins are reversibly dissociable component systems. However, not all proteins in their native state are composed of molecules of this order of magnitude. For example the greater part of the material in cow's milk from which crystalline lactal-bumin and lactoglobulin can be prepared has a molecular weight probably not exceeding one thousand (150). If the milk serum is treated with a neutral salt like ammonium sulfate, proteins with molecular weights ranging from 12,000 to 25,000 are obtained.

Therefore, it appears there are protein precursors of relatively low molecular weight which can easily be changed either by the organism in vivo, or by physical and chemical manipulation in vitro, into products of greater particle size.

The stability of proteins as a function of the pH can also be measured by means of the ultracentrifuge. A number of proteins have been prepared which within certain pH ranges were found to be monodispersed (osmotically homogeneous). They can be divided into two large groups: those with molecular weights from 34,500 to 208,000, and those with molecular weights from one to six million. The proteins of the first group appear to be divided into four classes with molecular weights of 1, 2, 3, and 6 times 34,500. Protein molecules containing more than one group of weight 34,500 dissociate into molecules of fewer groups when the pH of the surrounding solution is raised above a certain level. Thus proteins of 208,000 (6×34,500) split up into particles of one-half, one-third, and one-sixth of the original molecule, but never into particles of one-quarter or one-fifth. The latter observation is in concordance with the fact that proteins of molecular weight four and five times 34,500 at or near the isoelectric point have not been encountered. At sufficiently high alkalinity, all soluble proteins are supposed by some investigators to have this unit molecular weight, i.e., 34,500.

Furthermore it appears that the integer sub-molecules of any specific protein are electrochemically identical. On the other hand, chemically different proteins having molecules of the same weight are electrochemically different. However, if the pH of the solution is raised or lowered sufficiently, a point is reached at which the protein molecule is broken down into fragments of low molecular weight and differing electrochemically. Even this decomposition can in many cases be reversed if the pH of the mixture is brought back to within the stability range of the protein. This breaking down of a protein into substances of lower molecular weight recalls to mind the reversibly dissociable component systems of Sörensen.

(10) X-Ray Studies of Protein Structure (Astbury)

The study of the internal structure of simple crystals by the x-ray method of W. H. and W. L. Bragg was soon extended to the more complex materials of inorganic origin. These investigations indicated that the crystalline state is much more common than had been generally supposed. In fact, it is said that the crys-

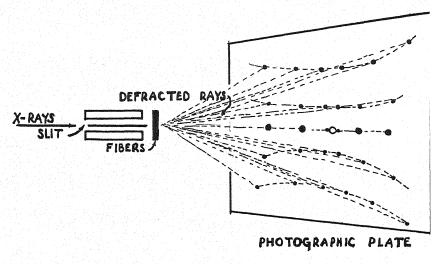


Fig. 1. Technique for Obtaining an X-ray Diagram.

talline state is the most general in solid matter. X-ray investigation of cellulose, starch, inulin, silk fibroin, fibrin, etc., suggested that

A Portion of the Cellulose Chain, Compound of Glucose Residues:

these substances are constructed from crystalline particles (crystallites). Little success, however, was achieved with proteins until

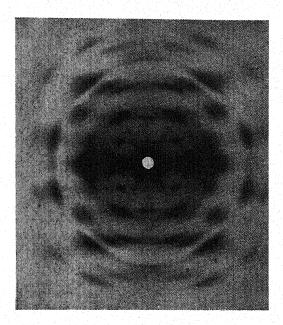


Fig. 2. Fiber Photograph of Native Cellulose. (Astbury, W. T., Chimie Indust. Spec. No., 31, 186, (1934).)

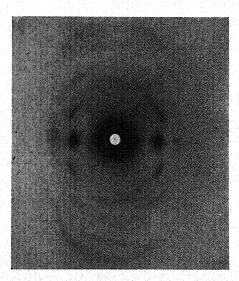
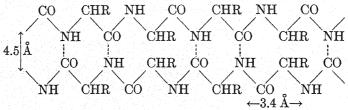


Fig. 3. Fiber photograph of natural silk fibroin. (Astbury, W. T., and Woods, H. J., Proc. Roy. Soc. London, 232 A, 394, (1933).)

after the solution of the problem of the structure of cellulose fibers. On the basis of these investigations, Meyer and Mark (153) suggested that silk fibroin is constructed from polypeptide chains

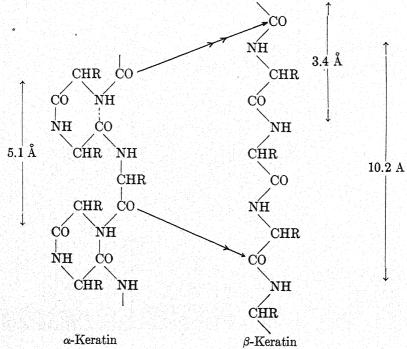
Diagram of a Portion of a Silk Fibroin Micelle Illustrating the Fully Extended Peptide Chains, (α-Keratin type) and the "Backbone" Spacing.



(The R-groups should be visualized as existing at right angles to the plane of the paper.)

lying roughly parallel to the fiber axis and clinging together to form long, thin, crystalline or pseudo-crystalline bundles or micelles. They suggested that the glycine and alanine rests of fibroin follow each other alternately in the polypeptide chains of fibroin and that

The Molecular Rearrangement Involved in Stretching. The Conversion of the α - into the β -Keratin form.



(Note that the rings are essentially open diketopiperazine rings. The R groups are at right angles to the plane of the paper.)

if the polypeptide chains were fully extended, each peptide rest should occupy a length along the fiber axis of about 3.5 Å $(3.5\times10^{-8}$ cm.). This hypothesis was in excellent agreement with the pattern and dimensions of the x-ray photograph obtained from silk fibroin. However, other x-ray photographs of hair, collagen,

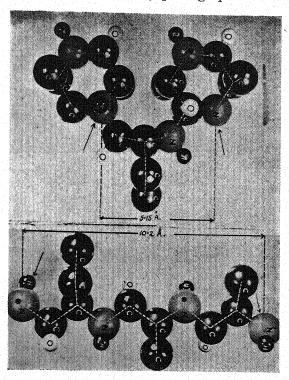


Fig. 4. The α-β-keratin Transformation Illustrated with Atomic Models. (Astbury, W. T., and Woods, H. J., Trans. Roy. Soc. London, 232 A, 333, (1933).) (See also Astbury, W. T., Woods, H. J., Speakman, J. B., Stoves, J. L., and Whelwell, C. S., J. Text. Inst., 28, 394 (1937); Astbury, W. T., Chem. Weekblad, 33, 778 (1936); Astbury, W. T., and Dawson, J. A. T., J. Soc. Dyers and Col., 54, 6 (1938); Astbury, W. T., Trans. Faraday Soc., 1937, p. 3; Astbury, W. T., Compt. rend. Lab. Carlsberg, 22, 45 (1938); Preston, R. D., and Astbury, W. T., Proc. Roy. Soc. London, 122 B, 76 (1937).)

etc., did not yield to this simple reasoning. The main features of all these fibrous structures are possibly analogous—that is, they are all based on long chain molecules aggregated into sub-microscopic crystalline bundles lying roughly parallel or spirally inclined at an approximately constant angle to the fiber axis¹³—but the configura-

¹³ Corey and Wyckoff (153a) state that this idea "probably contains much truth. The objection that must be raised against it is this, that x-ray data from which it has been developed are not adequate to show whether it is true or false."

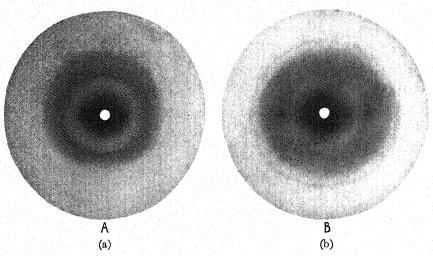


Fig. 5. X-ray Photographs of α - and β -keratin. Note the differences of the pattern at the equitorial plane. The inner ring is to be associated with the side-chain spacing, the outer with the backbone (lateral) spacing, of the polypeptide chains. (a) = α -keratin, (b) = β -keratin.

(Astbury, W. T., and Woods, H. J., Trans. Roy. Soc. London, 232 A, 333, (1933).)

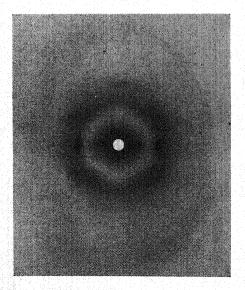


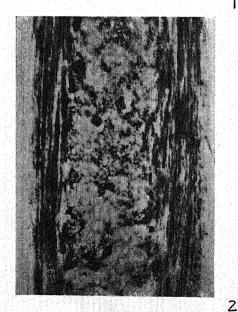
Fig. 6. Human hair at 100 per cent extension. (Astbury, W. T., and Woods, H. J., Trans. Roy Soc. London, 232 A, 333, (1933).)

tion of the polypeptide chains was not always as in natural silk. In fact fibroin was rather the exception than the rule. This discrepancy was surmounted by the experiments of Astbury (154, 155, 156, 157, 158), who showed that an intramolecular transformation

A Diagrammatic Representation of the Side-Chain or Grid Linkages Between Parallel Polypeptide Chains. The figure illustrates the grid for β -keratin. The α -form is derived from this by folding the paper into a series of regular folds which leave the side-chains still parallel to the plane of the paper.

(The main chains (–CO·NH·CH·CO·NH·CH–) should be visualized as existing at right angles to the plane of the paper. The variations in the distance between parallel main chains is caused by differences in the type linkage, e.g., the salt bond between the ϵ -amino group of lysine and the δ -carboxyl of glutamic acid is pictured as spreading the chains further apart than the –SS– linkage of two eysteyl rests.)





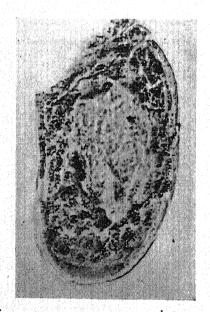


Fig. 7. Photomicrographs of Keratins.

(1). Cross Section of a Whalebone Filament. Observe that the cells are flattened and have been laid down tangentially in coaxial cylinders.

(2). Longitudinal Sections of a Pigmented Wool Fiber Showing the Fibrous Nature of the Cells.

(Astbury, W. T., and Sisson, W. A., Proc. Roy. Soc. London, 150 A, 533, (1935).)

of hair keratin takes place on stretching. It was long known that hair could be reversibly stretched if moistened. This increase in length was indicated by changes in the x-ray photograph which showed that reversible intramolecular transformation of the fiber substance, keratin, had taken place. Astbury calls normal keratin, α -keratin and the completely denatured, longer modification, β -keratin. The basis of this remarkable degree of elasticity of mammalian hairs is that of a molecular spring, an observation appar-

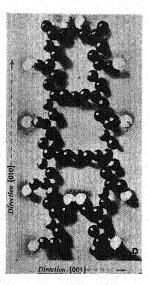


Fig. 8. Model Illustrating Unequal Cross Linkages. (Astbury, W. T., and Woods, H. J., Trans. Roy. Soc. London, 232 A, 333, (1933).)

ently of profound consequence in the study, not only of hairs, but of movement in general. In fact, the α - β -type of intramolecular transformation accompanying the stretching of myosin and of muscle protein itself in situ has been successfully carried out. It appears that the myosin molecule is normally in a folded configuration endowed with inherent long-range elasticity (159).

The accompanying diagrams taken from Astbury's papers indicate the nature of the linkage between these peptide chains. β -keratin is pictured as containing fully extended peptide chains, while in the α -modification the chains are so compressed as to form hexagonal rings. These rings if closed would give diketopiperazines. These scleroproteins, which from their gross physical nature appear to be fibrous substances, contain polypeptide chains which run lengthwise along the fiber. These chains are linked side by side by

virtue of electrostatic covalent and possibly primary valency linkages. The average separation of the main chains in the plane of the side chains (the plane of the grid) varies from 9.6 to 11.5 Å units. This observation could be expected because the average side chain separation of polypeptide chains which, although they might be roughly of the same magnitude, should vary from protein to protein depending on amino acid composition.

Some possible side chain linkages can be formulated as follows:

- (1) The uniting of peptides through the disulfide of cystine, i.e., the two cysteine rests are located in two different peptide chains.
- (2) In salt or ester linkage between acidic and basic amino acids; for example a salt formed between the δ -carboxyl of glutamic acid and the ϵ -amino group of lysine.
 - (3) By covalent linkages, esters, etc.

In the β -keratin type of protein the grids remain piled one on top of another with the main chains parallel and separated by a distance of 4.5 ± 0.1 Å. The greater constancy of this (backbone) spacing is to be expected as the polypeptide grids are united to each other only by the covalent linkages of the adjoining carbonyl and imino groups.

The original studies on the fibrous nature of the scleroproteins have been extended to other proteins, which, as we learn from the work of Svedberg usually are globular in solution. Astbury, together with Lomax (160), Dickinson and Bailey (161), has recently presented evidence indicating that these globular proteins during the course of heat or chemical denaturation assume a fibrous structure typical of that found in β -keratin. However, to the writer it appears from the above mentioned studies, that polypeptides as they are present in actively metabolizing centers, may be organized in a loosely knit grid formation including in the grid not only polypeptides but possibly also lipids and other cell constituents (cf., the cholesterol-phosphatide-protein complex of Mâcheboeuf). These complex grids can be easily changed either by the organism or by physical and chemical manipulations into the globular proteins composed of reversibly dissociable component systems. These soluble proteins are in turn converted into the microcrystalline fibrous substances having a lower degree of solubility. Such a transformation is indicated by the formation of the relatively insoluble crystalline fibrin from soluble fibrinogen. Fibrinogen, in turn, may not pre-exist in the plasma, but is a relatively easily precipitable co-precipitation system which is removed without difficulty from the living plasma. Another example of the degeneration (161) of

proteins by natural processes is given by the transformation of soluble protein of the cells of the hair follicle into the very insoluble degenerated protein, keratin.

The process of denaturation appears then to be the result of a firmer coalition of the peptide chains and an *increase in the degree* of crystallinity of the protein. Thus before denaturation the peptide chains seem to cling together chiefly by means of their active side chains (e.g., glutamic acid plus lysine). However, after denaturation, this coalition is markedly strengthened by virtue of a coalescence of the main chains by way of their backbone (carbonyl and imino) linkages.

(11) The Cyclol Theory of Protein Structure (Wrinch)

Wrinch (162, 163, 164) suggested that proteins are made up of closed polypeptide chains consisting of amino acid residues. The hexagonal folding of polypeptide chains, open or closed, permits the

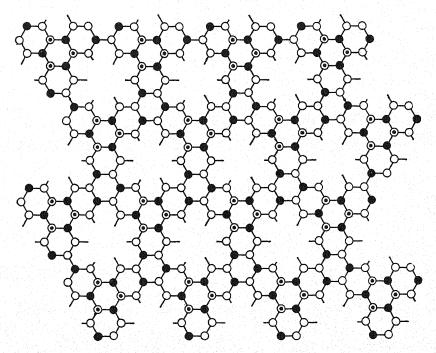


Fig. 9. The Cyclol Pattern.

● =N. \bigcirc = C(OH), hydroxyl upwards. \bigcirc = C(OH), hydroxyl downwards. \bigcirc = CHR, direction of side chain initially outwards. \bigcirc = CHR, direction of side chain initially upwards. The median plane of the lamina is the plane of the paper. The lamina has its "front" surface above and its "back" surface below the paper.

(Block, R. J., Yale J. Biol. Med., 9, 445 (1937).)

THE 'CYCLOL 6' MOLECULE

construction of molecules containing even hundreds of amino acid residues in orderly arrangement and provides a characteristic pattern for the complex protein molecule. The diazine rings comprising the cyclol molecule are believed to be formed either by lactamlactim rearrangement or by supposing that the =CO and =NH groups have united through a hydrogen bond.

The cyclized polypeptides can form hexagons lying roughly in one plane, the thickness of which is one amino acid residue. Since all naturally occurring amino acids are of the l-series this fabric is dorsiventral, having a front surface from which the side-chains emerge, and a back surface free from side-chains. Both front and back surfaces carry groups of hydroxyls $[=C(OH) \cdot N =]$ normal to the surface in alternating hexagonal arrays. These cyclol fabrics may be used to build space-enclosing molecules and multilaminate molecular aggregates of three dimensions. If this theory is substantiated, it implies that some native proteins, including the socalled globular soluble proteins, are space-enclosing, lace-like, polyhedral structures. This hypothesis explains, in a way, the nonrandomness found by Svedberg to be characteristic of the molecular weights of proteins. The reversible disaggregation of globular proteins into smaller molecules (molecular weight 34,500), which is affected by simply changing the pH of the solution, can also be explained by assuming that the multiple cyclol polyhedra (Figs. 10 & 11) are broken down into single units. Thus it appears that the synthesis of proteins can be considered as a process of cyclization of polypeptides. This idea implies that protein synthesis would be facilitated when the appropriate groups are in the imide form. This deduction from the cyclol hypothesis gains support from the investigations of Bergmann that under the influence of proteolytic enzymes, the keto form of the peptide linkage is changed to the imide form (cf. Footnote 8).

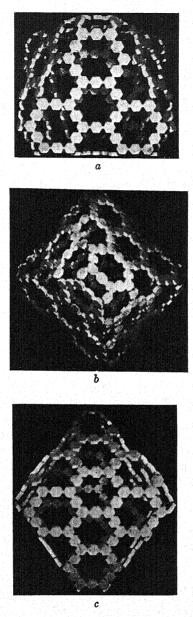


Fig. 10. The C₂ Molecule Comprising 288 Residues. (Wrinch, D. M., *Philos. Mag.*, Ser. 7, 24 (Supplement) 940 (1937).)

In spite of the fact that the acceptance of the cyclol theory offers a ready and apparently logical explanation of many facts in protein chemistry, nonetheless it must be remembered that little experimental work has appeared either for or against this hypothe-

sis. There is no evidence available to the reviewer's knowledge that these diazine rings are capable of hydrolytic fission by proteolytic enzymes but the information obtained from investigations on diketopiperazines (cf. above) are probably not applicable to these structures.

In summary, it appears that proteins are potentially both chain systems and ring systems, and whether they assume the form of chains or rings, depends on their external environment.

6. SUMMARY

1. Proteins consist of chains of 15 to 300 amino acids united by primary valences through their amino and carboxyl groups.

2. The basic amino acids—arginine, histidine, and lysine—may play an especially important rôle in the genetic development of

tissue proteins.

- 3. The actively metabolizing proteins of living tissues are probably bound to each other via their polar side chains and to the nonprotein cellular constituents by secondary valences. It is possible that these labile proteins have a loosely knit fibrous organization which readily breaks down on chemical manipulation to yield the isolated proteins, the composition and properties of which depend, in a measure, on the reagents employed in their preparation.
- 4. Isolated soluble proteins often exist in globular form having a molecular weight of 34,500 or some simple multiple thereof.

7. CONCLUSION

In this chapter, the reviewer has attempted to gather together some of the many chemical and physical facts which throw light on the structure of the proteins. Any theory of protein structure must account for a number of observations among which are:

1. Proteins are largely, if not entirely, composed of amino acid residues joined together in peptide linkage. There are relatively few "free" amino and carboxyl groups. 2. Soluble proteins differing widely in particle size can often be converted into equimolecular substances by mere adjustment of the pH of the solvent. 3. Many native proteins are globular in solution. 4. Proteins, even though they may differ widely in chemical constitution, show a similarity of behaviour which suggests some general plan in the arrangement of the amino acid residues. 5. The amino acid residues are definitely oriented in space, as indicated by physical, immunological, and enzymic reactions.

The reader must bear in mind that no one of the several protein hypotheses herein described entirely accounts for these and other known facts. Therefore no single hypothesis should be accepted at present without qualifications. However, it is quite possible that with the development of our knowledge, an entirely adequate theory will be formulated which will explain all the facts relating to protein behaviour. Ultimately to succeed in this formulation, thorough and careful interpretation and correlation of the already existing data as well as properly planned new investigations are required.

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CHAPTER VIII

MOLECULAR WEIGHTS OF THE PROTEINS

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1. INTRODUCTION

The problem of determining the molecular weights of the proteins has been approached from a number of standpoints. As will be brought out below, only a few methods have yielded results of significant value. Due to the large size of the protein molecule, it is impossible to obtain solutions of a reasonably high molecular concentration. This limits the number of experimental avenues of approach to the study of this problem.

The successful means of investigating the molecular weights of the proteins may be divided into two general classes. The first is based upon the chemical principle of stoichiometry, i.e., on the chemical composition of the protein, or on the combination of the protein with various reagents. The second depends upon the relation between certain physical chemical properties of protein solutions and the sizes of the molecules.

2. THE CALCULATION OF MOLECULAR WEIGHT FROM CHEMICAL COMPOSITION

One of the important methods of obtaining the molecular weight is based on either the elementary composition or the amino acid content of the protein. If a given element is a molecular constituent of a compound, it is evident that at least one atom of this element must be present in each molecule. Consequently the molecular weight cannot be less than the mass which contains one gram equivalent of the element. The compound may contain more than one atom of the element, in which case the molecular weight is an integral multiple of the value calculated on the assumption that only one atom is present. These relations may be embodied in the following equation:

Molecular Weight =
$$n \frac{\text{Atomic weight of element}}{\text{Per cent of element in the compound}} \times 100$$
 (1)

where n represents the number of atoms of the element present. For purposes of illustration let us take, as a concrete example, the calculation of the molecular weight of glutamic acid. The nitrogen content of this amino acid is found to be 9.52 per cent. The molecular weight is

$$n \frac{14}{9.52} \times 100 = n 147.$$

If there is only one atom of nitrogen present in the molecule, as happens to be the case in this instance, the value of n is unity, and 147 is the true molecular weight. This value, 147, is called the minimal molecular weight because the molecular weight cannot be smaller than this amount. If the molecular weight is larger, it must be some multiple of this number. In order to evaluate n, thus making it possible to calculate the true molecular weight from the value of the minimal molecular weight, data from some additional source are necessary. These are ordinarily obtained by measuring one of the colligative properties of solution of the compound.

It is apparent that this method is quite refined since it is independent of the properties of the compound, such as association and dissociation, when the compound is in solution. The method depends solely on the accuracy of the analysis for the particular element. However, the evaluation of n becomes increasingly difficult as its value increases. If, for example, the percentage of carbon had been chosen for the above calculation, the minimal molecular weight found would have been only one-fifth of 147, or about 30. Errors in the freezing point depression method or other methods used for the evaluation of n might be in error by an amount as large as the value of this minimal molecular weight. Such an error could lead to a value of 4 or 6 for n instead of the true value, 5. From this example it is apparent that if the method is to be useful it is necessary to choose an element or a certain group which can be determined accurately, and which is present in the molecule in a relatively small amount.

If it is feasible to use the analytical values of more than one element which enters into the composition of a compound, further valuable information in fixing the molecular weight is obtained. To illustrate this, suppose a compound contains 16 per cent of sulfur and 17.5 per cent of nitrogen. On the basis of the sulfur content, the minimal molecular weight is $32/16 \times 100 = 200$. From the nitrogen content the minimal molecular weight is $14/17.5 \times 100 = 80$.

It is readily seen that the actual molecular weight must satisfy the relationship,

$$n_1 \times 200 = n_2 \times 80$$

in which n_1 represents the number of sulfur atoms and n_2 the number of nitrogen atoms in the compound. By trial it is found that the above proportion is satisfied if one assumes that two atoms of sulfur and five atoms of nitrogen are present in the molecule. In this case the molecular weight is 400.

Applying the above considerations to the proteins, it becomes necessary to decide what elements may be used for the purpose of calculating minimal molecular weights. Using the criteria given previously, elements like carbon and nitrogen can be eliminated immediately since their percentages in the molecule are much too high, and the value of n becomes large and almost indeterminate. Sulfur, on the other hand, is found not only in most proteins, but it is frequently present in an amount small enough to be used for calculating the molecular weight. Since sulfur apparently may exist in the protein in various forms, such as sulfhydryl, disulfide, and thioether, it is possible by determining one of these special forms of sulfur instead of the total sulfur, to obtain data on the minimal molecular weight. Certain proteins are characterized by elements which are not present in most proteins. Thus hemoglobin contains iron, hemocyanin contains copper, and casein contains phosphorus. In some of these cases it is especially simple and satisfactory to use these elements for the purpose of calculating the minimal molecular weights provided they conform to the criteria given above. The phosphorus content of casein has not been useful in this connection for the reason that phosphates are a common contaminant of casein and the quantity of organically bound phosphorus is still somewhat in doubt. Moreover, since casein is not a homomolecular protein, its composition will depend upon the mode of preparation.

The outstanding example of the use of single elements for calculating a minimal molecular weight value is the case of iron in hemoglobin. Sulfur may also be used for this purpose. The data from the two sources are combined as was illustrated above. The hemoglobins were the first series of proteins to be studied in this manner. They have also been very extensively studied from other standpoints. Hüfner (1, 2) and Jacquet (3) have contributed the most complete data on the iron and sulfur content of hemoglobin obtained from various species. Their results, as well as those of vari-

ous other investigators, have been well summarized in the review of molecular weights of proteins by Cohn, Hendry, and Prentiss (4). Table I, which is reproduced from their article, summarizes the data on the hemoglobins with respect to their minimal molecular weights as calculated from their contents of iron, total sulfur, sul-

Table I
Minimal Molecular Weights of the Hemoglobins

	Method	Amount of constituent present	Weight combining or contain- ing 1 atom or molecule	Assumed no. of atoms or molecules	Minimal molecular weight
		per cent	gm.		
Horse	Iron content	0.335	16,669	1	16,669
	Sulfide sulfur content	0.190	16,878	1	16,878
	Sulfur content	0.390	8,223	2	16,446
Pig	Iron content	0.40	13,960	1	13,960
0	Sulfur content	0.48	6,681	$\tilde{2}$	13,362
Cat	Iron content	0.35	15,954	1	15,954
	Sulfur content	0.62	5,172	3	15,516
Ox	CO-combining capacity		16,721	2	33,442
	Iron content	0.336	16,619	2	33,238
	Sulfur content	0.45	7,127	5	35,635
	Sulfur content	0.48	6,681	5	33,405
	Arginine content	4.24	4,107	8	32,856
Fowl	Iron content	0.335	16,669	2	33,338
	Sulfur content	0.86	3,729	9	33,561
\mathbf{Dog}	Iron content	0.336	16,619	3	49,857
	Sulfide sulfur content	0.335	9,573	5	47,865
	Sulfur content	0.568	5,646	9	50,814

(Cohn, E. J., Hendry, J. L., and Prentiss, A. M., J. Biol. Chem., 63, 721 (1925).)

fide sulfur, and, in one case, arginine as well as the carbon monoxide combining power.

Most of the data given in Table I were obtained a considerable number of years ago, and it seems quite possible that various inaccuracies may have been included in the analytical values. Certainly the methods for obtaining pure hemoglobin preparations at that time were not so refined as some which are now available. There are reasons for believing that hemoglobins from different species differ somewhat from each other, and it is possible that

these differences may be reflected in their molecular weights. Landsteiner and Heidelberger (5) demonstrated, by means of mutual solubility tests, that the oxyhemoglobins from two species, which were not too closely related, will dissolve in saturated solutions of each other as though they were being dissolved in water alone. This property is shown only by non-identical compounds. This may be considered as supporting the idea that hemoglobins from different species are dissimilar. The solubility work confirms earlier work of Heidelberger and Landsteiner (6) in which species differences in hemoglobin were demonstrated serologically. If we can accept the idea that the chemical composition of hemoglobin from different species differs, it would not be surprising that such differences might also be reflected in their molecular weights. However, no such variation has vet been observed. Adair (7) concludes from osmotic pressure measurements that the molecular weight of hemoglobin from man, horses, oxen, and sheep is the same.

The various hemocyanins afford another interesting case of minimal molecular weights deduced from their content of a special element. This type of protein serves some of the lower forms of life in the same capacity that hemoglobin serves the higher forms, i.e., as a respiratory pigment. The hemocyanins contain copper. The copper content may be determined with considerable accuracy. The amount present in hemocyanin is sufficiently small to be of value in calculating the minimal molecular weight. The earliest analyses of copper in hemocyanin appear to have been made by Griffith (8). The analytical values for copper in the hemocyanins from different animal species have been carefully surveyed by Redfield, Coolidge, and Shotts (9). The data which they have compiled and the minimal molecular weights calculated from them are given in Table II.

Not all of the values cited are of equal reliability. It is probable that the older values are somewhat in error. However, the values show the magnitude of the minimal molecular weights, and demonstrate rather definitely that no single value may be assigned to all of the hemocyanins. This has been confirmed by studies which have been made on this group of proteins with the ultracentrifuge.

Some proteins have no elements in amounts small enough so that they can be used in calculating molecular weights. In some proteins even the amount of sulfur is too great to be of much use in this connection. Egg albumin serves as a good example of a protein which contains so much sulfur that the total percentage composition with respect to this element is of little significance. According to the data of Calvery (10), egg albumin contains 1.36 per cent of sulfur which gives a value of 15 or 16 molecules of cystine in one molecule of the protein. Such a value of n is too high for the accurate evaluation of the molecular weight.

The use of the percentage content of certain amino acids in minimal molecular weight calculations has been of the greatest

Table II

Minimal Molecular Weights of Hemocyanins Calculated from their Content of Copper

Species	Per Cent Copper	Minimal Molecular Weight	
Cancer	0.32	19,866	
Homarus		18,697	
Sepia		18,697	
Octopus vulgaris	0.38	16,729	
Helix pomatia	0.25	25,428	
Watferit		21,920	
Limulus polyphemus		36,700	

(Redfield, A. C., Coolidge, T., and Shotts, A. M., J. Biol. Chem., 76, 185 (1928).)

value for supplementing the calculations based on elementary composition. The method of calculating molecular weight on the basis of amino acid composition is exactly the same as that used when the basis of the calculation is the elementary composition. If a certain amino acid is contained in the protein molecule, it is evident that at least one molecule must be present in the combined state. If the number of such molecules is small, the calculation and the evaluation of n may be carried out as previously described in the case of the elements. The analyses, of course, must be capable of considerable accuracy. The use of amino acids has the advantage over the use of an element in calculating molecular weight since it is not likely that the protein will be contaminated by an amino acid. The particular amino acids which most generally fill the above requirements, and consequently are of the greatest utility, are tyrosine, cystine, and tryptophane. These three amino acids may be determined quite accurately and, with the possible exception of tyrosine, the amounts present in proteins are usually quite small. Our knowledge of the molecular weight of glutenin, a cereal protein obtained from wheat, rests almost entirely on the content of these three amino acids, together with the content of β -hydroxyglutamic acid. A summary of the data on glutenin has been included in the review of Cohn, Hendry, and Prentiss (4). Table III is reproduced from this article. The data illustrate the use of the amino acid content in calculating minimal molecular weights. The cystine results which are given in Table III are not concordant and have not been used in the calculation.

Glutenin is one of the few proteins, the knowledge of whose molecular weight rests on such incomplete data as the above. It serves, however, as an excellent example of how much information regard-

Table III

Minimal Molecular Weight of Glutenin

Method	Amount of constituent present	Weight combining or contain- ing 1 atom or molecule	Assumed no. of atoms or molecules	Minimal molecular weight
	per cent	gm.		
Tryptophane content	1.68	12,149	3	36,447
β-Hydroxyglutamic acid content	1.8	9,061	4	36,244
Tyrosine content	4.5	4,024	9	36,216
Cystine content	1.80	13,344		
Cystine content		15,397		

(Cohn, E. J., Hendry, J. L., and Prentiss, A. M., J. Biol. Chem., 63, 721 (1925).)

ing molecular weights may be obtained from the analysis for a very few amino acids.

Due largely to the efforts of Vickery and Leavenworth (11) who have based their methods on the older silver method of Kossel and Kutscher (12), the hexone bases may now be determined with considerable accuracy. The calculations of minimal molecular weights of proteins on the basis of the analyses of the hexone bases have proven useful in a large number of cases. Mention has already been made of the use of the arginine content for calculating the molecular weight of hemoglobin (see Table I). All three of the hexone bases have been used to calculate the minimal molecular weight of hemocyanin from *Limulus*. In fact, the use of the hexone bases for calculating molecular weights is so general that the only reason for not including them with tyrosine, tryptophane, and cystine as members of the most important class of amino acids which are used for this purpose is that the number of molecules of each of the hexone bases derived from the molecule of protein is generally con-

siderably larger than the number of molecules of the three amino acids mentioned.

In addition to β -hydroxyglutamic acid which was mentioned in connection with glutenin, other amino acids which have been used for minimal molecular weight calculations are phenylalanine, aspartic acid, proline, oxyproline, and leucine.

So much analytical data on the content of amino acids and of special elements in proteins have accumulated that it is not feasible to attempt to quote them in entirety. In spite of the quantity of data, considerable uncertainty still exists regarding the exact composition of all of the proteins with respect to most of their constituents, and, as has already been pointed out, the majority of the data are not useful for the calculation of minimal molecular weights.

3. THE CALCULATION OF MOLECULAR WEIGHT FROM COMBINING WEIGHTS

The law of the constant composition of chemical compounds, together with Dalton's law of multiple proportions which was enunciated about 1802, serve as the fundamental bases upon which rest the estimations of molecular weights or, more precisely, the equivalent weight of proteins obtained from values of their combining weights. As applied to proteins, these laws lead to the conclusion that any reagent which combines stoichiometrically with the protein will do so in definite amounts, i.e., simple molecular proportions. The weight of protein combined with an equivalent of reagent will be a simple fraction of the molecular weight. This fraction, which we shall call 1/m, where m is the number of groups in the protein molecule which react with the reagent in question, is entirely analogous to the fraction 1/n which was used in the preceding section. Suppose that a protein contains five free carboxyl groups in the molecule. A gram mole of protein will then react with five equivalents of base. To illustrate this concretely, let us assume that the amount of base which combines with 10 grams of protein, for example, is 1.47×10^{-3} equivalents. This means that 10 grams is 0.00147 of the equivalent combining weight of the protein. The latter quantity is equal to 10/0.00147 or 6,800. The value of the equivalent combining weight of the protein, which is analogous to the minimal molecular weight, is one-fifth of the true molecular weight. The latter quantity is then 34,000. In general, the value of m, the number of reactive groups in the molecule of protein, is not known but can be approximately evaluated by other methods just as was described for the evaluation of n in the preceding section. Since the proteins are amphoteric electrolytes, titration with acids is equally useful for obtaining the equivalent combining weight.

Both acid and base have the very great advantage of being capable of combining with nearly all of the proteins. On the other hand, their use is limited due to the fact that the total number of groups in the protein molecule which will react with acid or base is usually large, thus making the accurate evaluation of m difficult.

The maximum amount of acid or base which will combine with a given weight of protein is usually estimated from titration curves carried out with the aid of the hydrogen or other electrodes. The nature and the interpretation of the titration curves are discussed in chapters XI and XIII. The equivalent weights calculated from the electrometric measurement of the acid- and base-combining capacities of a few representative proteins are reported in Table IV.

The base- or acid-combining capacity of a protein may also be determined by titration with acidic or basic dyes (see Chapter XIII). This method was applied quantitatively by Chapman, Greenberg, and Schmidt (13). They titrated proteins with acidic dyes, but did not attempt to calculate molecular weights from their data. However, the values which they obtained for the combining capacity of proteins appear to be among the most accurate which are available, and consequently may be used in obtaining minimal molecular weights. The type of combination which takes place between protein and dye is the same as that which takes place between protein and acid or base. The data obtained by titrating proteins with dyes are more accurate than when the protein is treated with acid or base since the protein-dye compound is insoluble, and therefore a more definite endpoint is reached in the titration. Rawlins and Schmidt (14, 15) extended this work to include the use of basic dyes, and thus obtained values for the base binding capacity as well. Table V summarizes the results obtained in these studies. The data have been used to calculate the equivalent combining weights of several proteins.

Some difficulty was encountered in determining the capacity of proteins to combine with basic dyes. Apparently the high alkalinity at which it was necessary to work hydrolyzed part of the acid amide groups. Consequently these data are less reliable in calculating equivalent combining weights than those obtained by the use of acidic dyes.

The solubility of a protein in base or in acid may, in some in-

Table IV

Equivalent Weight of Proteins Calculated from their Acid- and
Base-Combining Capacities

Protein	Maximal acid combining capacity per gram of protein	weight of protein	Refer- ence	Maximal base combining capacity per gram of protein	weight of protein	Refer- ence
	Eq. ×105			Eq. ×105		
Egg albumin	87	1150	(1)	105	950	(2)
Denatured egg albumin.	83	1130	(3)	72	1390	(3)
Serum albumin	155	645	(4)	130	770	(2)
Serum albumin	72	1375	(3)	70	1430	(3)
Gelatin	96	1090	(5)	70	1430	(6)
Deaminized gelatin	44	2200	(7)			_
Gliadin	_			30	3333	(8)
Casein	-			160	625	(8)
Thymus histone	108	930	(9)	149	670	(9)
Myogen	135	750	(2)	128	780	(2)
Edestin	134	750	(10)		_	_
Hemoglobin	149	667	(11)	95.5	1048	(11)
Zein		-	-	30	3400	(11)

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The values given in this table have been selected from those which appear to be the most reliable. For a more complete summary, see Pauli, W., and Valkó, E., Kolloid-Chemie der Eiweisskörper, Dresden and Leipzig, 1933, p. 41.

stances, be used to determine the combining capacity near the isoelectric point. This is a very different quantity from the maximum base- or acid-combining capacity. In the case of an isoelectric protein which is soluble in pure water to a negligible extent, but whose salts with base or acid are soluble, the amount of acid or base combined to form such salts is determined by measuring the increase in solubility with known additions of base or acid. The amount of base or of acid combined is, however, much less than the maximum amount which the protein is capable of binding. It has generally been assumed that only one or two groups in the protein react at a pH near the isoelectric point.

Osborne (16) was apparently the first to apply the method of solubility to the determination of the minimal molecular weights of

Table V

Combining Weights of Proteins from their Titration Values with Acidic and Basic Dyes

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Protein	Acidic dye combining capacity per gram of protein	Equivalent combining weight of protein cation	Basic dye combining capacity per gram of protein	Equivalent combining weight of protein anion
Gelatin	Eq. ×10 ⁵	961	Eq. ×10 ⁵	1429
Casein		1000	210	476
Edestin	. 157	637	70	1429
Fibrin	. 145	690	168	595

(The data in this table have been compiled from the work of Chapman, L.M., Greenberg, D. M., and Schmidt, C. L. A., J. Biol. Chem., 72, 707 (1927); and Rawlins, L. M. C., and Schmidt, C. L. A., J. Biol. Chem., 82, 709 (1929).)

proteins. He studied the solubility of edestin in both sodium hydroxide and hydrochloric acid solutions. The molecular weight was calculated to be 14,260 on the assumption that a molecule of edestin combines with only one molecule of hydrochloric acid under the conditions of the experiment. Cohn and Hendry (17) have made measurements on casein, which is very slightly soluble in water but whose sodium salt is quite soluble. The minimal molecular weight derived from these solubility measurements is 2100. Cohn, Hendry, and Prentiss (4) report similar studies on the serum globulins. Assuming that one mole of globulin combines with one mole of base, the average value of 14,240 was obtained for the molecular weight.

With the respiratory proteins, such as hemoglobin and hemocyanin, certain gaseous reagents, namely, oxygen and carbon monoxide, have been used to better advantage than acids or bases for the purpose of estimating the combining weight. Hemoglobin, which furnished a good example of the use of elementary analysis for obtaining minimal molecular weights, also serves as an especially interesting example of the use of this combining weight method. Hüfner (2) found experimentally that one gram of hemoglobin combines with a maximum of 1.34 milliliters of oxygen, measured under standard conditions. This remains the accepted value. He also obtained the same value for the combination with carbon monoxide. It has been pointed out by Peters (18) and others that the amount of gas taken up by hemoglobin at complete saturation is always parallel to the iron content of the protein. The seat of the combination of a gas with hemoglobin is therefore attributed to the presence of iron in the molecule. This necessarily means that determining the maximum gas absorptive capacity of hemoglobin is equivalent to determining the content of iron and applying the method of chemical composition to the determination of the minimal molecular weight. If one gram of hemoglobin combines with 1.34 milliliters of oxygen or carbon monoxide under standard conditions, the weight of hemoglobin which will combine with one mole of either of these gases is given by the proportion

$$\frac{22,400}{1.34} = \frac{\text{Equivalent combining weight}}{1} = 16,717$$

It is evident that this value is the equivalent combining weight. This value checks well with the minimal molecular weight of hemoglobin as calculated from the content of iron, sulfur, and certain amino acids.

Redfield, Coolidge, and Montgomery (19) investigated the combination of oxygen with the hemocyanins from nine different species. They found in all cases, approximately within the experimental limits of error, that one atom of oxygen is bound for every atom of copper present in hemocyanin. They did not attempt to correlate the oxygen combining capacity directly with the actual weights of protein used, so that it is impossible to calculate directly the equivalent combining weight. Since they proved that the reaction is stoichiometrical and depends on the copper content of the hemocyanin, the equivalent combining weight must be the same as the minimal molecular weight calculated from the content of copper (9). As in the case of the methods which are based on chemical composition, it is desirable that the data on equivalent combining weights be combined with those from other available methods in order to obtain an accurate evaluation of m.

The efforts which have been directed to the calculation of the

molecular weights of proteins from their elementary composition, from their amino acid content, and from their combining weights have, on the whole, proved disappointing. The probable values of the molecular weights of a number of proteins, compiled by Cohn, Hendry, and Prentiss (4) from all evidence available at the time, are given in Table VI. At present the data given in this table are chiefly of historical interest. They represent the state of our knowledge on this subject up to 1925. Since then, through the application of certain physical chemical methods, particularly that of the ultra-

Table VI

Estimated Values of the Minimal Molecular Weights of Proteins

Protein	Minimal molecular weight
Gelatin	. 10,300
Zein	. 19,400
Gliadin	. 20,700
Hemocyanin, Limulus	22,700
Bence-Jones' protein	
Edestin	
Hemocyanin, Octopus	. 33,500
Egg albumin	
Glutenin	
Fibrin	. 42,000
Serum albumin	
Hemoglobin	. 50,000
Serum globulin	
Casein	

(Cohn, E. J., Hendry, J. L., and Prentiss, A. M., J. Biol. Chem., 63, 721 (1925).)

centrifuge, a vast amount of reliable data has been accumulated. Excepting those proteins on which information from physical chemical methods was also available, the values assigned for the molecular weights given in Table VI have proven to be largely in error, as will be seen in the following sections.

The difficulties due to the presence of impurities in the proteins, to imperfections in the methods of analysis, and to fixing the values for n have already been discussed. One of the main difficulties, however, was almost totally unappreciated. This was due to the fact that many of the proteins studied were not single, homomolecular substances, but were mixtures or protein-protein compounds. Obviously the application of stoichiometrical principles to mixtures can hardly produce anything but results of dubious accuracy.

4. THE DETERMINATION OF MOLECULAR WEIGHTS FROM OSMOTIC PRESSURE MEASUREMENTS

(1) General Considerations. Among the most important generally available methods for determining molecular weight are those which are based upon the so-called colligative properties of solutions. Included in these properties are lowering of the vapor pressure, elevation of the boiling point, lowering of the freezing point, and osmotic pressure. All of these properties depend directly upon the influence which the solute exerts on the thermodynamic activity of the solvent. This influence, in dilute solution, is almost directly proportional to the number of particles, molecules, ions, or micelles which are dissolved in a definite amount of the solvent. Consequently, if a known weight of solute is dissolved and the effect on any one of the colligative properties is measured, the magnitude of this effect is approximately a measure of the number of particles into which the solute is divided.

However, with the exception of the osmotic pressure, estimation of the colligative properties cannot easily be carried out on protein solutions, as can readily be seen from the following considerations. One gram mole of a solute dissolved in 1000 grams of water depresses the freezing point of the water approximately 1.86° provided that the solute forms a molecularly dispersed solution. Consequently, a comparatively dilute solution, in terms of molecular concentration, will depress the freezing point sufficiently so that it can be measured with a fair degree of accuracy. However, if a protein which has a molecular weight of, for example, 34,000, were the solute, and if it were soluble to the extent of about 1 per cent by weight (a value which is an approximation to the solubility of many proteins), the freezing point would be depressed only about 10/34,000 × 1.86°, or about 0.00055°.¹ A value of this magnitude cannot at present be measured with a sufficient degree of accuracy to be reliable. Similar considerations hold for the use of vapor pressure data except that, in this case, the sensitivity of the method is considerably less than is the freezing point depression. Measurements of the elevation of the boiling point do not differ greatly in their sensitivity from lowering of the freezing point. In addition,

¹ In the course of their study on the freezing point lowering of the case in ates of the alkaline elements, Robertson and Burnett (20) made the following observation regarding the minute effect of the protein itself. When the concentration of base was kept constant, increasing the quantity of case in dissolved in it in the proportion of 8 to 5 did not alter the freezing point depression in any appreciable degree. Their method was sensitive to a change of $\pm 0.0025^{\circ}$.

boiling would, in most cases, coagulate or denature the protein. The only method which is based on colligative properties of solutions, and which is at present applicable to protein solutions with any ease or accuracy, is that of osmotic pressure. The reason that this method alone gives sufficient sensitivity is that one mole of an undissociated solute dissolved in 1000 grams of water exerts an osmotic pressure at 0° of approximately 22.4 atmospheres. Thus, in the case of the protein considered above, the osmotic pressure would be approximately 10/34,000×22.4, or about 0.0066 atmosphere, or a pressure of 5.016 mm. of mercury. Since the osmotic pressure is usually measured by means of a column of water instead of mercury, this pressure is equivalent to about 68.22 mm. of water, an amount which is easily measured with the required accuracy.

Another important reason that favors the osmotic pressure method and militates against the use of the other methods which have been mentioned is the effect of the presence of impurities of low molecular weight. It is extremely difficult to prepare proteins free from all contaminating substances. In most of the methods which are dependent on the colligative properties of solutions, these contaminants, because of their low molecular weights relative to that of the protein, contain many unit particles which exert a large effect on the magnitude of the measurement. On the other hand, since membranes which are permeable to compounds of low molecular weights but impermeable to proteins are employed in the osmotic pressure method, the smaller molecules will distribute themselves in such a manner as not to contribute to the osmotic pressure.

(2) Theory of Osmotic Pressure. In order to understand fully the basis of molecular weight determinations of proteins by means of osmotic pressure measurements, it is desirable that the fundamental theory of osmotic pressure be reviewed. It appears that the soundest and most general interpretation of this phenomenon is obtained from the standpoint of "escaping tendency," a function which quantitatively correlates osmosis with the other colligative properties.

Lewis and Randall (21) define the term, "escaping tendency," as the tendency of a material to pass from one phase to another. Thus if water and water vapor are in equilibrium, the "escaping tendency" of the water is the same in both phases, i.e., the tendency for the water to pass from the liquid to the gaseous state is the same as the tendency of the water vapor to pass to the liquid phase.

If one constructs a cell with a semipermeable membrane, i.e., a membrane which will allow water, for example, to pass through it, but will not allow the passage of some material dissolved in the water, and fills this cell with a solution and immerses it in water, the system will consist of two phases separated by a semipermeable membrane. In the system described, water will pass through the membrane and into the solution contained in the cell until the hydrostatic pressure which is established will exactly balance the tendency of the water to enter the cell. The outside solution need not be water but may be any liquid mixture whose constituents are capable of penetrating the membrane. The phenomenon of osmosis will be observed whenever the concentration of the solute in the two phases is different, the water moving from the more dilute to the more concentrated solution. By concentration in the osmotic sense is meant the concentration of all solute particles whether they be molecules, ions, or micelles.

Under any fixed set of conditions water has a certain escaping tendency. If any material is dissolved in the water, some sort of restraint is placed on the water molecules, e.g., by attraction for the solute molecules, and the escaping tendency is lowered by some corresponding amount. This lowering of the escaping tendency may be measured by the change produced in any one of the colligative properties. For example, the vapor pressure is lowered, since the tendency of the liquid to pass to the vapor phase is lowered. For the same reason the boiling point is raised, since it now requires a greater rise in temperature to make the vapor pressure of the water equal to the atmospheric pressure. Likewise, the tendency for water to pass into the solid phase is correspondingly lowered and hence the freezing point is depressed. The effect of greatest magnitude produced by a lowering of the escaping tendency of the solvent is on the osmotic pressure. In an osmotic cell, when the escaping tendency of the water in the solution is lowered to a value less than that of the water outside of the semipermeable membrane, water will pass from the water phase to the solution phase through the membrane. The escaping tendency of the water in the solution inside the membrane will be continually increased by the increase in both the water concentration and the increase in pressure, until the escaping tendencies are again equalized on the two sides of the membrane. At this point the system is in equilibrium and the hydrostatic pressure exerted by the solution gives a measure of the osmotic pressure. In other words, the osmotic pressure is the hydrostatic pressure which has to be impressed upon the inside solution to equalize exactly the escaping tendency of the solvent on the two sides of the membrane and to prevent its flow in either direction.

A satisfactory mathematical treatment for the theory of osmotic pressure has been derived strictly from thermodynamic considerations. According to Lewis and Randall (21) the equation which is so obtained and which is strictly accurate is

$$RT \ln \frac{f_1^0}{f_1} = \int_{P^0}^{P} -\overline{V}_1 dP$$
 (2)

where f_1^0 is the fugacity of the pure solvent, f_1 is the fugacity of the solvent of the solution, \overline{V}_1 is the partial molal volume of the solvent in the solution, P is the pressure exerted on the solution at osmotic equilibrium, and P^0 is the pressure exerted on the pure solvent. By fugacity is meant a quantity which is a measure of the escaping tendency and which is defined as "equal to the vapor pressure when the vapor is a perfect gas." In general, it may be regarded as an ideal or corrected vapor pressure (21). The partial molal volume, \overline{V}_1 , is defined by the equation,

$$\overline{V}_1 = \frac{\delta V}{\delta n_1} \tag{3}$$

where V is the total volume of a mixture and n_1 the number of moles of the solvent in the mixture, and where all independent variables such as temperature and pressure are held constant.

In dilute solutions having a small osmotic pressure, \overline{V}_1 becomes practically constant and equal to V_1 , the molal volume of pure solvent. Then, as an approximation for dilute solutions, equation (3) reduces to

$$RT \ln \frac{f_1^0}{f_1} = V_1(P - P^0) \tag{4}$$

Using the designation P_0 for the osmotic pressure $(P_0 = P - P^0)$, equation (4) becomes

$$RT \ln \frac{f_1^0}{f_1} = V_1 P_0 \tag{5}$$

A further simplification of the osmotic pressure equation which is widely used may be obtained in the following manner: in dilute solutions the fugacity ratio $f_1/f_1^0 = N_1 = (1 - N_2)$, where N_1 and N_2

are the mole fraction of the solvent and solute, respectively, of a binary solution. On substituting this relation in equation (5) (21, p. 231), there is obtained

$$P_0 = -\frac{RT}{V_1} \ln N_1 \tag{6}$$

On expanding the term $\ln N_1$ in a series there is obtained

$$-\ln N_1 = -(N_1 - 1) + 1/2(N_1 - 1)^2 - 1/3(N_1 - 1)^3 + \cdots$$
 (7)

For use in dilute solutions all but the first member of this series are ignored. Substituting the first member of the series in equation (6) gives

$$P_0 = -(N_1 - 1) \frac{RT}{V_1} \tag{8}$$

But since $-(N_1-1)=N_2$, the equation for the osmotic pressure may be written as

$$P_0 = N_2 \frac{RT}{V_1} \tag{9}$$

This equation is slightly different in form, but is quite analogous to the equation derived by van't Hoff (22), viz.,

$$P_0V = nRT$$
 or $P_0 = CRT$ (10)

where P_0 is the osmotic pressure, V is the volume of solution, n is the number of moles of solute, R is the gas constant in literatmospheres, T is the absolute temperature, and T is the concentration (equivalent to T in T in equation of van't Hoff shows that the osmotic pressure is directly proportional to the concentration, a relation which corresponds to Boyle's law for gases. Also the osmotic pressure is directly proportional to the temperature. This is similar to Gay-Lussac's law for gases. The obvious implication is that a mole of dissolved solute exerts exactly the same pressure as a mole of perfect gas when confined in the same volume, with the exception that the conditions which allow this pressure to be manifested are different.

 2 The gas law constant, R, has the following values in the different pressure units which are widely used in osmotic pressure work.

Liter-atmosphere per degree 0.08207 Liter-mm. Hg per degree 62.37 Liter-mm. H₂O per degree 848.3 Later findings by a considerable number of investigators demonstrate that the above laws of osmotic pressure obtained by van't Hoff hold moderately well for dilute solutions, but that in higher concentrations they are not rigidly applicable. The development of the van't Hoff equation depends on the assumption of the existence of a perfect solution. Most solutions behave as perfect solutions only at a very great dilution. The data are nearly always obtained on solutions whose concentrations are such that this equation is not strictly applicable. Just as van der Waals corrected the gas law equation for the volume actually occupied by the gas molecules, Sackur (23) and later Porter (24) have shown, by applying a similar correction for the volume occupied by the solute molecules to the osmotic pressure equation, that an equation is obtained which fits the experimental data better. The equation which they derived is

$$P_0(V-b) = nRT \tag{11}$$

where b is the factor which corrects for the volume of solute molecules. This factor was not found to be constant, but varies with the temperature and the concentration. It is therefore somewhat empirical. Equation (11) has found important use as the basis of an empirical method for treating the departure of the osmotic pressure from ideal behavior with increasing concentration of the solution.

(3) Application to Protein Solutions. In applying the general theory of osmotic pressure to proteins, it must be borne in mind that proteins are electrolytes. Corrections for the influence of ionization of proteins are based on the Donnan theory of membrane equilibria. However, since the simple equations which were derived by Donnan (see Chapter XIV) apply only to ideal solutions, and since the proteins do not form ideal solutions, their application is still not completely satisfactory. The development of a theory for the interpretation of osmotic pressure data of proteins along exact thermodynamic lines is due chiefly to Adair (7, 25, 26). In this theory the Dalton conception of partial pressures of a gas mixture is applied to the osmotic pressure. This is expressed by the equation

$$P_0 = P_p + P_i \tag{12}$$

in which P_0 is the total observed osmotic pressure, P_p is the partial osmotic pressure of the protein, and P_i is the partial osmotic pressure due to the diffusible ion difference on the two sides of the membrane. In order to calculate the molecular weight, it is necessary to know the value of P_p . The difficulty arises in evaluating P_i .

As is brought out in Chapter XIV, P_i , which is the Donnan osmotic pressure, is a function of the membrane potential of the system. Adair's effort was to find an exact means of evaluating P_i from the measured membrane potentials. His results may be summed up in the following equations:

$$P_{p} = P_{0} - P_{i} = RTg_{p}m_{p} = 10RTg_{p} \frac{C_{p}}{M}$$
(13)

In this equation R is the gas law constant, T the absolute temperature, g_r the osmotic coefficient of the protein, m_r the molar concentration of the protein in grams per liter of solvent, C_r the concentration of the protein in grams per 100 ml. of solvent, and M the molecular weight of the solvent.

To calculate P_i , the following equation is employed:

$$P_i = RT \int_0^\mu m_p n_p d\mu \tag{14}$$

In equation (14) m_p is the molar concentration, and n_p the mean valence of the protein ions. Thus the term $m_p n_p$ represents the equivalent concentration of the protein ions. The term μ has the value (EmF)/RT in which Em is the membrane potential and F the Faraday number. If Em is expressed in millivolts and the constants in the equation are numerically evaluated, then at 0°, $\mu = Em/23.5$.

The main difficulty in applying the above equation for P_i is to evaluate n_p , the mean valence of the protein ions. According to Adair and Robinson (27, 28) $m_p n_p = \mu J$ is an approximation. Here J is the ionic concentration, i.e., the sum of the concentration of the ions in the dialysate multiplied by the squares of their valences. In other words, it is twice the ionic strength as defined by Lewis and Randall.

On substituting the above terms in equation (14) there is obtained

$$P_{i} = RTJ \int_{0}^{\mu} \mu d\mu = 1/2RTJ\mu^{2} = 1/2RTJ \left(\frac{Em}{23.5}\right)^{2}$$
 (15)

As was previously explained, J stands for the ionic concentrations and Em for the membrane potential. For further details of the development of these equations, the reader is referred to the original publications.

The results obtained through the application of osmotic pressure

measurements to the determination of the molecular weights of proteins will now be examined. For the readings to be of any value it is necessary that the system be in osmotic equilibrium. This may be established by the application of the following criteria: (a) the hydrostatic pressures should remain constant over quite a long period of time; (b) the same final pressure value should be attained

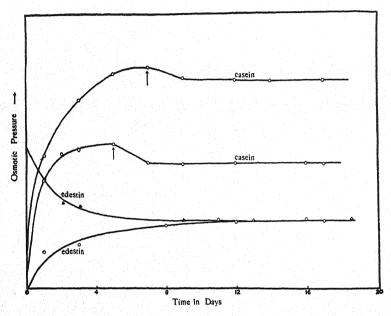


Fig. 1. Rate of attainment and constancy of the osmotic pressure equilibrium. The edestin curves, showing how equilibrium is attained starting either from a higher or lower pressure, have been shifted along the ordinate axis so as to coincide. as they represent edestin at different concentrations. The arrows of the casein curves indicate the time at which stirring of the solution ceased.

(Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197 (1930).)

on starting with initial hydrostatic pressures either greater or less than the osmotic pressure; (c) finally, the results should be reproducible when different solutions of the protein and different membranes are used. A graphical illustration of the application of certain of these criteria, taken from the results of Burk and Greenberg (29), is shown in Fig. 1.

According to Adair (30), the factors which may influence the osmotic pressure of protein solutions containing different amounts of electrolytes are: changes in the state of aggregation of the proteins, the unequal distribution of ions across the membranes, and the effect of attractive and repulsive forces between the ions and molecules present in the solution upon the thermodynamic activity of the protein.

(4) The Work of Sörensen on Osmotic Pressures of Protein Solutions. Sörensen (31, 32) was the first to refine adequately the experimental technique of osmotic pressure measurement and he was also the first to realize all of the criteria which must be applied

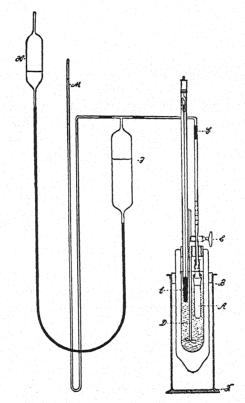


Fig. 2. Apparatus for measuring osmotic pressure. (Sörensen, S. P. L., Proteins, New York, 1925, p. 33.)

in order that data suitable for the calculation of the molecular weights of proteins may be obtained. The type of osmometer which he employed is shown in Fig. 2. It contains a mercury manometer for producing a counter pressure to the osmotic pressure in order to minimize dilution. A simple type of osmometer without this device, introduced by Burk and Greenberg (29), is shown in Fig. 3.

Sörensen carried out a very careful study of the osmotic pressure of crystalline egg albumin using solutions of ammonium sulfate as the solvent. He emphasized the necessity of giving an exhaustive definition of the composition of the solution and of assuring the constancy of this composition during the time of experimentation. Due to these precautions, he was able to show positively that two egg albumin solutions having the same composition invariably exhibited the same osmotic pressure, a point previously unproven and not supported by the then available data. Another factor emphasized by Sörensen is the fact that ordinary changes in the degree



Fig. 3. Apparatus for determining the osmotic pressure of protein solutions. The mechanical rocking arrangement is not shown.

(Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197 (1930).)

of dispersion or association of a protein are negligible in comparison with the influence of ionization. Because proteins are amphoteric electrolytes, capable of forming salts with either acids or bases, the osmotic pressure will vary in a manner which is dependent on the amount of salt thus formed. Since the amount of acid or base combined is dependent upon the value of the pH, control of this factor is of extreme importance.

In connection with the relation of pH to the osmotic pressure of proteins, it should be pointed out that at some particular pH values, a given protein either combines with no acid or base, or its combination with acid is exactly equal to its combination with base. This point is not exactly the isoelectric point when the iso-

electric point is different from exact neutrality, as is probably always the case. The isoelectric point is defined as that pH value at which the protein carries exactly as many positive as negative charges. All known proteins have an excess of acidic over basic groups, or vice versa. Most of the common proteins are predominantly acidic having more ionizable acidic than basic groups. Thus if a pure protein is placed in pure water or in neutral salt solution, and the ionization of the water itself is neglected, the protein will dissociate more hydrogen than hydroxyl ions or vice versa, and will carry an excess of negative or of positive charges. In most cases the predominant charge will be negative and therefore the protein cannot be in the isoelectric condition. It is nevertheless in an uncombined state, and would here exert the nearest to its true osmotic pressure. This point has been called the isoionic point by Sörensen, Linderström-Lang and Lund (33).

Whereas the isoelectric point of a protein is readily determinable. the isoionic point is determined only with considerable difficulty. Usually no distinction is made between the two points, or else the deviation from the isoelectric point is estimated by one of several possible methods. Sörensen, by a rather involved method, estimated the pH value at which the ratio of distribution of ammonium sulfate in the two solutions is equal to unity, and found it somewhat higher than the isoelectric point. This is necessarily the case with the isoionic point of egg albumin or any other protein which has the isoelectric and isoionic points lying on the acid side of neutrality. At the point where the ratio of distribution of salt between the two solutions is equal to one, the observed osmotic pressure must obviously be due entirely to the protein, unless the protein has a relatively large effect on the activity of the added salt. The magnitude of such an effect is difficult to evaluate. Sörensen assumed that it is not very large. In order to minimize errors arising from deviation of the protein from the isoionic point, and from inequality of the concentrations of salt in the two solutions, it is desirable to employ dilute protein solutions in relatively concentrated salt solutions. This, in fact, is the method usually adopted for accurate measurements, rather than the apparently simpler method of using soluble proteins at their isoelectric points without added salts.

The work of Sörensen was one of the first attempts to treat osmotic pressure data in a sound theoretical manner. Even now his technical methods might well serve as a model for accurate osmotic pressure investigations. The value which he obtained for the molecular weight of egg albumin is approximately 34,000. Using the same technique he later reported the following values for the molecular weights of the serum proteins: serum albumin 45,000 and serum globulin 80,000 to 140,000. He suggested that the first value for serum globulin represents pseudo-globulin, the second, euglobulin. It is to be noted that Sörensen's treatment of his data was still inadequate, though it was an attempt to apply the general principles which were later dealt with more adequately by Adair and others.

(5) The Work of Adair and Others on the Osmotic Pressures of Protein Solutions. An investigation of the osmotic pressure of egg albumin, using the method of Adair, was carried out by Marrack and Hewitt (34). Instead of using ammonium sulfate solutions as solvents, as did Sörensen, other salt solutions were used, viz., acetate buffer alone and in conjunction with sodium chloride. The osmotic pressure curve which they obtained for egg albumin coincided well with the curve obtained by Sörensen for this protein in ammonium sulfate solutions. The presence or absence of sodium chloride in the acetate buffer had no effect on the curve.

For the purpose of molecular weight calculation, the osmotic pressure of a protein must either be measured at the point of minimum combination with acid or base, or the measurements must be corrected to correspond to that condition. Two methods are available for doing this. Measurements are carried out on solutions in which the pure protein is dissolved in water, or it may be dissolved in either a solution of neutral salt or in a buffer system. Estimation of the osmotic pressures of the proteins in buffered solutions or in the presence of a neutral salt is preferable for the following reasons. Many proteins are negligibly soluble at the isoelectric point. Osmotic pressure measurements obviously cannot be carried out unless the isoelectric protein can be dissolved in a salt solution. An isoelectric protein will dissociate a considerable number of hydrogen or hydroxyl ions unless the isoelectric point happens to correspond with exact neutrality. For this reason, the true osmotic pressure of a protein is not obtained at the isoelectric point, but rather in the neighborhood of the isoionic point. In an extensive study of the molecular weight of hemoglobin, Adair carried out measurements in the absence of salts, only as a confirmatory check, and particularly to determine whether or not the salt caused a change in the state of aggregation as had been previously claimed. He obtained no such effect. Hüfner and Gansser (35) had also used solutions of horse and ox hemoglobin to which no salt had been added in their measurements. Adair pointed out that these investigators apparently were using a salt of hemoglobin instead of isoelectric hemoglobin. This would reduce the apparent molecular weight to at least one-half of the true value.

Table VII
Osmotic Pressures of Hemoglobin at Different Hydrogen ion Concentrations

The osmotic pressure is expressed in terms of the pressure in Hg per 1 per cent of protein at 0°. The experimental temperature was 23°. (Adair, G. S., *Proc. Roy. Soc.*, *London*, 109 A, 292 (1925).)

pН	P_{0}	Grams Hb per 100 c.c. solution	pН	P ₀	Grams Hb per 100 c.c. solution
5.0 5.4 6.5 6.7* 6.8 6.8	21.5 13.4 3.2 2.4 2.4 3.5	0.74 1.20 1.20 3.70 6.20 6.20	6.8 6.8† 7.2 9.6 10.2	4.5 6.8 5.0 15.6 21.4	4.00 0.97 1.20 3.67 1.20

^{*} Iso-electric point—minimum osmotic pressure.

The data of Adair on the osmotic pressure of hemoglobin in the absence of salts are given in Table VII. The measurements were carried out on dialysed sheep hemoglobin. The conductivity and pH of the dialysed solutions were also measured. It was observed that the lowest values of the osmotic pressure corresponds to the lowest conductivity value. This was at the isoelectric point.

The change of osmotic pressure with change of protein concentration is illustrated in Fig. 4. It was found that the curve in the figure can be represented by the equation,

$$P_0(V-1.68) = 255 \tag{16}$$

in which P_0 is the osmotic pressure and V = 100/C Hb = ml. of solvent per gram of hemoglobin. CHb = grams of hemoglobin per 100 ml. of solution. This equation is of the type developed by Sackur (23) and by Porter (24). The molecular weight of isoelectric hemoglobin was calculated to be about four times the value of the minimal molecular weight of 16,700 (calculated from the iron content). This indicates that there are four iron atoms in the molecule

[†] Denaturation observed.

and the true molecular weight is four times the minimal molecular weight. Perhaps the most important conclusions to be drawn from this work are (a) that aggregation of this protein does not take place with increased concentration, and (b) that salts do not cause aggregation of hemoglobin, since the molecular weight of the electrolyte-free protein agrees with that obtained in salt solutions.

Adair (25, 26) also has made extensive measurements on the osmotic pressure of hemoglobin in the presence of various salts and

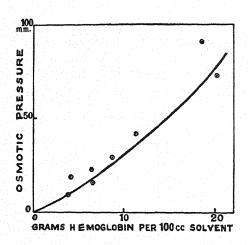


Fig. 4. The change of osmotic pressure with protein concentration of dialyzed sheep hemoglobin.

(Adair, G. S., Proc. Roy. Soc. London, 109 A, 292 (1925).)

buffer mixtures. These include varying concentrations of sodium or potassium chloride buffered with bicarbonate or phosphate mixtures. A considerable range of variation of concentration of protein, salt, and pH was studied in order to determine the effect of each of these factors. The osmotic pressure data were correlated with the membrane potentials and the distribution of the diffusible ions. The interpretation of the data was carried out in accordance with the equations which have already been discussed. In Table VIII and Fig. 5 are given illustrative data from Adair's work.

Adair found that at low protein concentrations the osmotic pressure was, within wide limits, independent of the salt concentration. Under these conditions the partial osmotic pressure of the hemoglobin solution remained nearly constant between pH 6.8 and 7.8. From the work in salt solutions, a final value of 68,000 for the molecular weight of hemoglobin was obtained. This agrees well

TABLE VIII

The Partial Osmotic Pressures of Hemoglobin Ions at 0°, in Solutions Equilibrated with a Mixture Composed of 0.1 moles KCl, 0.0613 moles NaH_2PO_4 , and 0.00533 moles KH_2PO_4 , $pH = -log \ [H] = 7.8$

Grams protein per 100 c.c. so- lution CHb	Grams protein per 100 c.c. sol- vent cHb	$egin{array}{l} ext{Moles} \ ext{protein} \ ext{per 1000} \ ext{c.c. solvent} \ ext{} $	$egin{array}{l} Mem- \\ brane \\ potential, \\ milli- \\ volts \\ E_m \end{array}$	$\begin{array}{c} ext{Ob-} \\ ext{served} \\ ext{osmotic} \\ ext{pressure} \\ ext{$p_{ ext{obs.}}$} \end{array}$	differ-	Partial pressure of protein p_p	Provisional value p_i^*	Provisional value p_p^*
0.68	0.685	0.000103	-0.02	1.9	0.005	1.9	0.003	1.9
2.21	2.26	0.000338	-0.07	6.2	0.06	6.1	0.04	6.2
2.90	2.98	0.000447	-0.10	8.6	0.13	8.5	0.07	8.5
3.58	3.71	0.000555	-0.10	11.2	0.13	11.1	0.07	11.1
5.00	5.25	0.000787	-0.20	14.9	0.51	14.4	0.31	14.6
8.00	8.67	0.001300	-0.24	28.2	0.74	27.5	0.50	27.7
8.12	8.81	0.001320	-0.28	28.8	1.0	27.8	0.61	28.2
10.00	11.09	0.00166	-0.50	40.2	3.2	37.0	2.00	38.2
12.00	13.57	0.00203	-0.40	48.7	2.1	46.7	1.20	47.5
15.50	18.22	0.00272	-0.60	67.4	4.6	62.8	2.80	64.6
19.40	23.87	0.00357	-0.75	103.6	7.2	96.4	4.40	99.2
19.80	24.48	0.00367	-0.70	104.2	6.3	97.9	3.80	100.4
20.00	24.78	0.00371	-0.87	110.9	9.7	101.2	5.70	105.2
24.00	31.23	0.00468	-1.05	155.0	14.1	140.9	8.50	146.5
25.00	32.95	0.00493	-1.10	179.0	15.5	163.5	9.50	169.5
28.00	38.37	0.00574	-1.35	242.0	23.3	218.7	14.20	227.8
29.00	40.27	0.00603	-1.45	264.6	26.9	237.7	16.50	248.1
34.41	51.51	0.00771	-1.98	382.8	50.2	332.6	30.60	352.2

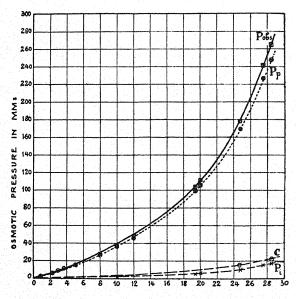
CHb=grams of dried hemoglobin prepared from sheep's blood, per 100 cc. solution. cHb is equal to CHb/1-0.00965 CHb, where 0.965 cc. is the effective volume of the protein in solution per gram of dry matter. $m_p = \text{cHb} \times 10/M$, where M, the molecular weight equals 66,800 $p_{\text{obs.}}$ = the observed osmotic pressure in millimeters of mercury at 0°. p_i = the ion pressure difference calculated by formula (15).

The values of m_p in column 3 are proportional to the values of the membrane potential, and it follows that $m_p n_p = k_1 u_1$, where k_1 is a constant, approximately 0.83. By substitution in formula (15), and by integration, $p_i = \frac{1}{2}RTk_1u^2 = 12.8 E_m^2$, where $E_m = 23.535 \ u =$ the membrane potential in millivolts at 0°. $p_p =$ the partial pressure of the protein. $p_p = p - 12.8 E_m^2$. $p_i^* =$ the provisional value of the ion pressure difference.

(Adair, G. S., Proc. Roy. Soc. London, 126 A, 16 (1929).)

with the previously found value in salt-free solution. No alteration in the state of aggregation of the hemoglobin was observed between pH 5 and 9 and in the presence of 0.01 to 4.0 molar concentrations of sodium or potassium chloride.

Of the proteins the hemocyanins apparently have the largest molecular size. Attempts to estimate this by osmotic pressure measurements have been made by Adair, Adair, Roche, and Roche (36). The molecular weight values found were of the order of 500,000 to 1,700,000 (see Table XI). While somewhat lower, these values approach the order of magnitude found by Svedberg and co-workers by use of the ultracentrifuge. With molecules as large as the hemocyanins, the osmotic pressure method reaches the limit of its accuracy.



CHb = Grams hemoglobin per 100 cc. solution.

Fig. 5. The osmotic pressure concentration curve of hemoglobin in the presence of potassium chloride and phosphate buffer at pH 7.8. $P_{\rm obs.}$ = observed osmotic pressure of hemoglobin in millimeters of mercury at 0°. P_p = partial pressure of the protein ions. P_i = diffusible ion pressure difference. C = diffusible ion pressure difference. CHb = grams dry hemoglobin per 100 cc. protein solution, in equilibrium with phosphate buffer mixture at pH 7.8. See original article for the calculations.

(Adair, G. S., Proc. Roy. Soc. London, 120A, 573 (1928).)

From a series of 27 measurements on the osmotic pressure of crystalline horse serum ablumin, Adair and Robinson (27) obtained a value of $72,000\pm3,000$ for the molecular weight. The curves of the osmotic pressure and of the membrane potentials of the system plotted against the protein concentration are shown, respectively, in Figs. 6 and 7. Albumin from ox and sheep serum gave a corresponding value of about 70,000. Human serum albumin was found by Roche, Dorier and Marquet (37) to have a molecular weight of 69,000.

Unfractionated horse, ox, and sheep globulin (27, 28) gave mean molecular weights of about 175,000. The results of seventeen determinations varied from 154,000 to 192,000. The state of aggregation of the proteins as they exist in the serum appeared to be the same as in purified preparations. A study of the osmotic pressure of the total serum protein before fractionation into albumin and globulin indicates that no compound of albumin with globulin exists in the blood as has been postulated at various times. The state of aggre-

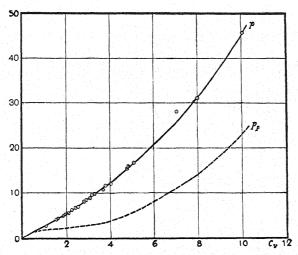


Fig. 6. Partial osmotic pressure of crystalline serum albumin at pH 7.4. Ordinate = pressure in mm. mercury at 0°. Abscissa = C_v = corrected concentration of protein in gm. per 100 cc. solvent. p = observed osmotic pressure. $p_p = p - p_i$ = partial pressure due to protein ions.

(Adair, G. S., and Robinson, M. E., Biochem. J., 24, 1864 (1930).)

gation of the proteins in the untreated serum appears to be the same as their state of aggregation in the purified state.

In this case, as in the case of hemoglobin, the application of Donnan's simple equations for membrane equilibrium was found to be wholly inadequate due to the deviation of the protein solutions from the ideal solution laws. Adair's work indicates in every case that the state of aggregation of the proteins studied is not changed by the presence of salts or by hydrogen ion concentration within the rather wide ranges studied.

(6) Osmotic Pressure Measurements of Proteins in Urea and Glycerol Solutions. An interesting extension of the osmotic pressure method for the determination of molecular weights of proteins which are not soluble in water in the isoelectric state was made by

Burk and Greenberg (29). It consists in the use of solvent materials of a non-ionic nature to bring the protein into solution. The use of non-ionic solvents avoids the difficult and often uncertain corrections which are necessary when the measurements are carried out in the presence of electrolytes. Another objective of the work of Greenberg and Burk was to determine whether proteins undergo changes in their state of aggregation with changes in the nature of the solvent.

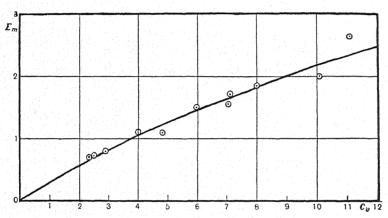


Fig. 7. Membrane potentials of crystalline serum albumin at pH 7.4. Ordinate=membrane potential in millivolts at 0°. Abscissa= C_v =corrected concentration of protein in gm. per 100 cc. solvent. Curve calculated from formula $C_v = 3.09E_m + 0.7E_m^2$.

(Adair, G. S., and Robinson, M. E., Biochem. J., 24, 1864 (1930).)

Burk and Greenberg used a 6.66 M urea solution as a solventy chiefly because it has an enormous dissolving power for proteins. As an alternative mixed solvent, 6.5 M glycerol solutions were used for comparison with urea. To the extent that the solvents were free from electrolytes, complications due to the effect of protein ions could be ruled out, thus requiring fewer corrections to be made than in the studies of Adair. To some extent this particular advantage was lost since it was found that the ionization of proteins as well as of the buffer solutions is altered by the presence of a high concentration of urea. This led to a change in the isoelectric point of the protein. It was therefore necessary to buffer the system to the isoelectric point. It was observed that the ionization of the acetate and phosphate buffer solutions was markedly altered by urea but not by glycerol. Accordingly the dissociation curves of these buffer systems in urea solution had to be redetermined. The

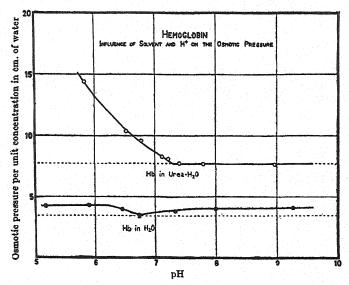


Fig. 8. The osmotic pressure of hemoglobin in different solvents. Dotted lines represent the minimum osmotic pressure in each solvent, corresponding to that of hemoglobin without the presence of a Donnan membrane equilibrium.

(Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197 (1930).)

use of buffers was necessary since when a protein was dissolved in urea solution without the addition of buffers, traces of base or acid found in the materials which were used produced a slight shift

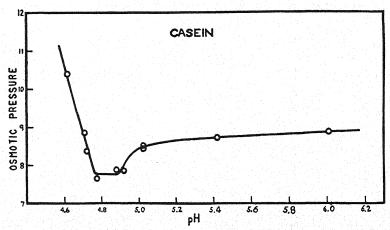


Fig. 9. Influence of the hydrogen ion activity on the osmotic pressure of casein. Location of the hydrogen ion activity at which the osmotic pressure is a minimum, (pH 4.78 to 4.92). Ordinates = osmotic pressure per unit of concentration in cm. of water.

(Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197 (1930).)

from the pH of the isoelectric point of the protein. However, when the protein was accurately adjusted to its isoelectric point, the presence of the buffer salts did not introduce any appreciable inaccuracy into the osmotic pressure measurements, since the buffer

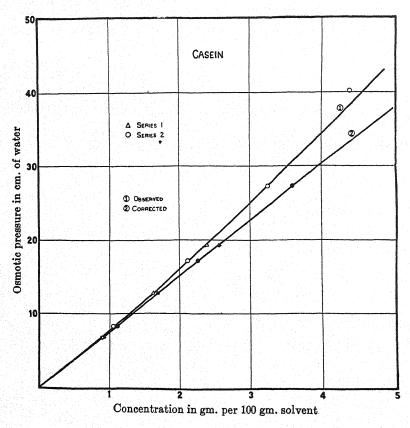


Fig. 10. Relationship between the concentration and the osmotic pressure of casein at the isoelectric point (pH 4.78 to 4.92). Curve 1 becomes linear (Curve 2) by correction of the concentration for solvation.

(Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197 (1930).)

salts were not combined with the protein and were freely diffusible through the membrane. The influence of change of pH on the osmotic pressure of proteins in urea solution is shown in Figs. 8 and 9.

The influence of change of concentration of the protein on the osmotic pressure was also studied. Figs. 10 and 11 show the results obtained on several proteins dissolved in 6.66 M urea solution. The observed osmotic pressure deviates upward from the theoretical straight line, the deviation increasing with increased concentration

of protein. Comparison of these curves with that of hemoglobin (Fig. 4) shows that this is a common phenomenon among proteins and, indeed, as has been pointed out many times, such a deviation is characteristic not only of proteins, but also of concentrated solutions of simple substances such as sugar. This type of osmotic pressure-concentration curve is due to the fact that, in general, even

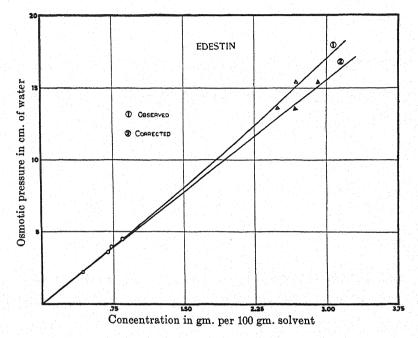


Fig. 11. Relationship between the osmotic pressure and the concentration of edestin at the isoelectric zone.

(Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197 (1930).)

dilute protein solutions deviate considerably in their behavior from an ideal solution. In order to correct for the departure from a linear relation between osmotic pressure and concentration, Burk and Greenberg employed the equation,

$$P_0 = K \frac{100C'}{100 - bC'} \tag{17}$$

where P_0 is the osmotic pressure, C' is the measured concentration in grams per 100 ml. of solvent, and K and b are constants. This equation is a modification of the one developed by Sackur and by Porter.

It has been known for some time that urea has a strong tendency to denature proteins. In the investigation of Burk and Greenberg, it was observed that egg albumin and hemoglobin are denatured. The osmotic pressure measurements on these proteins in urea indicated that the molecular weight of hemoglobin was halved by denaturation, while the molecular weight of the egg albumin was practically the same as that found in an undenatured aqueous solution by Sörensen (32) and by Svedberg and Nichols (38). Their values are about 34,000, whereas Burk and Greenberg obtained 36,000. In the case of hemoglobin, Burk and Greenberg obtained 34,300, whereas Adair found 68,000. To test whether this difference was due to the effect of change of solvent or to denaturation, Burk and Greenberg measured the osmotic pressure of hemoglobin in 6.5 M glycerol solution and obtained a value of 66,500 for the molecular weight. The facts lead to the conclusion that the low molecular weight of the hemoglobin in urea solutions is due to denaturation.

The findings of Burk and Greenberg have recently been confirmed by Steinhardt (102) with the aid of the ultracentrifuge. He found that native horse hemoglobin is totally dissociated into molecules of half the normal molecular weight by the presence of high concentrations of urea, acetamide, or formamide. According to Steinhardt and contrary to the observations of Burk and Greenberg, the change in molecular weight is unaccompanied by any evidence of denaturation. This discrepancy appears to be due to the fact that in the presence of oxygen the dissociated hemoglobin is very sensitive to denaturing influences. No effort was made to exclude oxygen in the experiments of Burk and Greenberg.

Huang and Wu (39) and Wu and Yang (40) made use of the technique of Burk and Greenberg to study the influence of denaturation on the state of aggregation of proteins. Their osmotic pressure measurements were not made with great precision, but were designed rather to detect large changes in the molecular weight of the protein if any denaturation occurred. Their data are given in Tables IX and X.

In agreement with Greenberg and Burk, no change in the molecular weight of egg albumin was observed on denaturation with urea or alcohol. On the other hand, as is seen from Table IX, denaturation by acid or alkali produced marked changes. The measurements with hemoglobin gave results which were dependent upon the animal species from which this protein was obtained. Ac-

cording to Wu and his co-workers, hemoglobin of the horse and ox undergoes a reduction in molecular weight, while that of the sheep and dog remains unaltered. In a careful study of the molecular

	TABI	E IX		
Molecular	Weight of Egg	Albumin in	Various	Solvents

Protein	Molecular weight	Denatured by
Egg albumin	$32,500 \pm 2,300$ $64,700 \pm 5,200$ $15,900 \pm 3,600*$ $34,900 \pm 2,800$ $32,300 \pm 2,700$	urea acid alkali alcohol in alkaline solution alcohol in acid solution

^{*} The low value is due to degradative changes as evidenced by liberation of ammonia, hydrogen sulfide, chromogenic substances, etc.

(Huang, T. C., and Wu, H., Chinese J. Physiol., 4, 221 (1931).)

Table X

Molecular Weights of Natural and Denatured Oxyhemoglobin,

Methemoglobin and Globin

Species	Protein	Natural protein in 65 per cent glycerine	Denatured protein in 40 per cent urea
Sheep	Methemoglobin Oxyhemoblobin Globin	65,700 66,600	69,400 65,600 63,000
Dog	Methemoglobin Oxyhemoglobin Globin	65,300 65,600	65,900 65,900 67,500
Ox	Methemoglobin Oxyhemoglobin Globin	65,100 64,900 68,000	39,200 37,700 34,300
Horse	Oxyhemoglobin Globin	62,300	35,800

(Wu, H., and Yang, E. F., Chinese J. Physiol., 6, 51 (1932).)

weight of the globins from various animal species, Roche, Roche, Adair and Adair (41) obtained 37,000 and 29,000 for the molecular weights of undenatured ox and horse globin, respectively. The molecular weights of the denatured paraglobin from the same species were 99,000 and 63,000, respectively. These results were ob-

tained with protein concentrations of about one per cent and at pH 6.5. The measurements on this protein appear to indicate that polymerization occurs as the protein concentration is increased. The work on the molecular weights of globin and of hemoglobin denatured by urea leads to the conclusion that the molecular formula of hemoglobin is Heme₄ Globin₂.

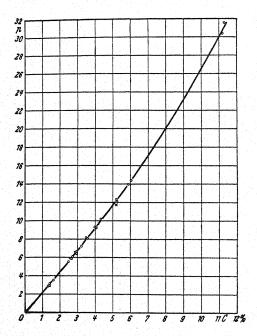


Fig. 12. The osmotic pressure-protein concentration of myogen at 277° absolute. Ordinates give osmotic pressure in mm. Hg. Abscissa represents myogen concentration in grams per 100 ml. Circles represent the measured pressures of purified myogen. Crosses represent measurements of fresh muscle press juice. The solid line corresponds to the equation

$$P_0 = \frac{\frac{g}{M} RT}{V - gS}$$

in which g is the concentration of protein, M is the molecular weight (81,000), R is the gas law constant, T is the absolute temperature, V is the volume used in terms of 0.1 liter as the unit, and S is an empirical factor assumed to correct for the protein hydration which has the value 0.00198.

(Weber, H. H., and Stover, R., Biochem. Z., 259, 269 (1933).)

Another interesting example of the influence of urea in altering the molecular size of a protein is offered by Weber and Stover (42). These authors observed that the muscle protein myogen has a molecular weight of 81,000 in aqueous solution, and 34,000 in urea. The change to this common value of 34,000 on denaturation would seem to be more than a mere coincidence. On the other hand, when ammonium thiocyanate solution was used as a solvent, a tremendous aggregation of the myogen took place. The molecular weight was estimated to be about 300,000. The osmotic pressure-protein concentration curve of myogen obtained by Weber and Stover is given in Fig. 12.

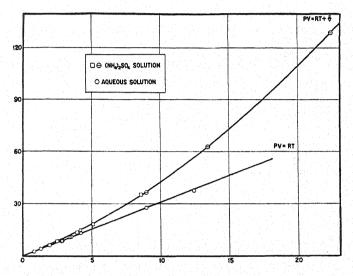


Fig. 13. Relationship of osmotic pressure to concentration of serum albumin, showing deviation of the osmotic pressure from the ideal law in ammonium sulfate solution. Upper curve: solvent, 0.74 N ammonium sulfate solution; lower curve: solvent, water (0.05 N acetate buffer solution). The ordinate represents the osmotic pressure measured in cm. of water; the abscissa, the concentration in gm. per 100 cc. of solvent.

(Burk, N. F., J. Biol. Chem., 98, 353 (1932).)

An interesting study on the stability of serum albumin from the horse under varying environmental conditions has been carried out by Burk (43). He obtained 74,600 for the mean molecular weight of this protein in aqueous solution. Serum albumin denatured by heat or urea was found to have essentially the same molecular weight in urea solution (73,000) as the native serum albumin. Measurements in 75 per cent glycerol solution yielded the same results as that of the native albumin in aqueous solution. Certain points which developed during the work of Burk are of great interest. Purified albumin in a dilute 0.05 M acetic acid-sodium ace-

tate buffer solution of pH 4.8 gave a concentration-osmotic pressure curve which conforms to van't Hoff's law. On the other hand, in 0.74 M ammonium sulfate solution there is a marked departure from this law. These results are brought out in Fig. 13. The osmotic pressure in ammonium sulfate solution may be expressed by the equation,

 $P_0V = RT + \frac{a}{V}$

in which the symbols have the usual meaning, a being a constant.

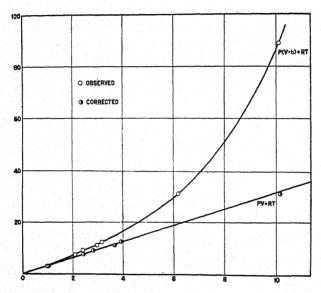


Fig. 14. Osmotic pressure of serum albumin in 6.66 M urea solution at the isoelectric point. Correction of the observed curve for deviation from ideality by Equation 6 yields a linear relationship of pressure to concentration. The ordinate represents the osmotic pressure measured in cm. of water; the abscissa, the concentration in gm. per 100 cc. of solvent.

The work of Burk offers an explanation for the discordant value of 45,000 for the molecular weight of serum albumin reported by Sörensen. Sörensen's data fit well on the curve as is shown in Fig. 13. Presumably Sörensen did not realize the large departure from ideal solutions which occurs in this system.

In urea solution serum albumin conforms to the same type of osmotic pressure-concentration curve that was observed by Burk and Greenberg. This is illustrated in Fig. 14. The discussion which has been given shows that there is a marked variation among different proteins with respect to the effect of denaturing agents on the molecular weight. The molecular weights of egg albumin and serum albumin are not changed when subjected to denaturation. On the other hand, the molecular weights of hemoglobin and certain other proteins are altered by denaturation³.

In connection with the problem of the effect of non-aqueous solvents on proteins the work of Cohn and Conant (44) with phenol is of interest. In checking up the suggestions that phenol produces a disaggregation of proteins into units of very low molecular weight, the above authors attempted, by use of the method of freezing point lowering, to measure the molecular weights of zein and gelatin dissolved in phenol. It was found that the results which had previously been reported were due to the presence of small amounts of water in the protein. When this disturbing factor was removed, the freezing point depressions in phenol were too small to be measured.

As has been brought out, of all the colligative properties, only the osmotic pressure is sufficiently sensitive to be of value in determining molecular weights of proteins. While there still exists some uncertainty as to the correct interpretation of osmotic pressure data, it is also probably true that only those proteins which have been studied carefully in this manner can have molecular weights assigned to them with any degree of confidence.

A summary of molecular weight data obtained from osmotic pressure measurements is given in Table XI.

5. SPECIAL PHYSICAL METHODS FOR DETERMINING MOLECULAR WEIGHTS OF PROTEINS

A number of methods which are based upon the relative rates at which protein molecules move through a solution under the influence of various forces have been developed for the determination of the molecular weights of the protein. The forces of special importance are thermal which give rise to diffusion, and gravitational or centrifugal which give rise to sedimentation.

³ In general, urea solutions and other denaturing solvents lead to the appearance of free —SH groups in proteins. According to Burk (47), the scisson of disulfide bonds of cystine, when the cystine residue is located in the interior of the protein, may account for the decrease in molecular weight which is shown by certain proteins in urea solution. If the cystine residue is in a peripheral position, its rupture would produce no measurable effect on the molecular weight. Burk also suggests that in some cases the disulfide linkage occurs in the protein as part of a ring structure. In such a case, —SH groups can be set free without any accompanying scisson of the protein molecule.

Table XI

Molecular Weights of Proteins Obtained by Osmotic Pressure Measurements

Protein	Molecular weight	Solvent	Refer- ence ²
Egg albumin	34,000	Ammonium sulfate	(32)
4 마시 사람들은 일반에 하다 보다고 ! !	36,000	6.66 M urea	(29)
Serum albumin (horse)	72,000	Buffered salt solution ³	(27)
	73,000	6.66 M urea	(43)
	74,000	7.0 M glycerol	(43)
	73,000	Heat denatured	(43)
Serum albumin (ox and sheep)	70,000	Buffered salt solution	(27)
Serum albumin (human)	69,000	Buffered salt solution	(37)
Serum globulin (horse)	175,000	Buffered salt solution	(27)
Serum globulin (horse)	150,000	Buffered salt solution	(45)
Serum globulin (horse)	173,000	6.66 M urea	(43)
Hemoglobin ¹	67,000	Water	(25)
리 경쟁 개호 노인을 보다는 경우가 제요	68,000	Buffered salt solution	(25)
	66,500	6.5 M glycerol	(29)
	34,300	6.66 M urea	(29)
Globin (ox)	37,000	Buffered salt solution	(41)
Globin (horse)	29,000	Buffered salt solution	(41)
Muscle protein (myogen)	81,000	Dilute buffer solution4	(42)
교통하다 기반하다 하다 하고 있는 것이다.	34,000	6.66 M urea	(42)
	300,000	Ammonium thiocyanate	(42)
Hemocyanin (Cancer)	500,000	Buffered salt solution	(36)
Hemocyanin (Limulus polyphemus)	700,000	Buffered salt solution	(36)
Hemocyanin (Helix pomatia)	1,700,000	Buffered salt solution	(36)
Hemerythrin (Sipunculus)	66,500	Buffered salt solution	(46)
Casein	33,600	6.66 M urea	(29)
Edestin	49,500	6.66 M urea	(29)
Gliadin	40,000	6.66 M urea	(47)
Zein	30,000	6.66 M urea	(47)
Excelsin	36,000	6.66 M urea	(47)
Amandin	30,000	6.66 M urea	(47)

¹ The molecular weights of carbon monoxide hemoglobin and oxyhemoglobin from the blood of man, sheep, ox, horse, and dog have the same value.

² The references refer to the list at the end of the chapter.

(1) Molecular Weights from Diffusion Measurements. All molecules are constantly moving under the influence of thermal forces. This gives rise to diffusion in systems in which a concentration gradient is set up. The rate of this diffusion will be in the inverse ratio to the size of the molecules. The most serious drawbacks to the use of diffusion in the past have been (a) the great length of time required for the diffusion of such large molecules as the pro-

³ This designation signifies that the measurements were carried out in buffered sodium or potassium chloride solution.

⁴ A low concentration of a buffer mixture was present to maintain a constant pH.

teins, (b) the difficulty of avoiding convection currents in the solution, and (c) the difficulty of arranging the experimental conditions so that the state of the system may be adequately defined for purposes of calculation.

In the past the difficulty of making diffusion measurements has been due to the complexity of the apparatus required to avoid or minimize convection currents. With the development by Northrop and Anson (48) of a simple and ingenious apparatus (see Fig. 18, Chapter XII) which eliminates this source of difficulty, it is now possible to obtain data from diffusion measurements which yield reasonably accurate values of molecular weights. The most essential portion of this apparatus is a membrane made from sintered glass having a thickness of about 0.5 mm. and pores of about 5 to 10 microns in diameter. Stirring, which would otherwise be essential to maintain uniform concentration, was avoided because the heavier solution would always sink to the bottom, thereby replenishing the protein solution at the surface of the membrane. With such a set-up, the only diffusion taking place is in the membrane itself and, since this area is constant, it can be defined and determined. Moreover, the speed of diffusion is relatively high because very high concentration gradients can be maintained. Convection currents will not interfere unless they take place within the membrane itself, which is scarcely possible inside the fine pores of such a membrane.

The diffusion coefficient is determined in the Northrop and Anson apparatus as follows. By definition, it is the quantity of material which will diffuse per second across a surface of one square cm. area under a unit concentration gradient. Hence

$$D = \frac{dQ}{A dt \frac{dc}{dx}} \tag{18}$$

In the equation, D is the diffusion coefficient and dQ is the quantity of diffusing material which passes across a plane of area A, in the time dt, and under a concentration gradient of dc/dx.

From the diffusion coefficient it is possible to calculate the molecular or micellar radius by use of the Einstein (49) equation,

$$D = \frac{RT}{N} \frac{1}{6\pi r \eta} \tag{19}$$

where R is the gas constant. It is equal to 8.3×10^7 ergs/degree /mole. T is the absolute temperature, N is the Avogadro constant, or 6.06×10^{23} , r is the radius of the particle including water of hydration, and η is the viscosity of the solution.

If the radius is calculated and the specific gravity is known, the molecular weight may be calculated from the relation, $M=4/3\pi r^3Nd$. All of the terms except d, which represents the density, have previously been defined.

In the case where a relatively concentrated solution is used on one side of the membrane and a pure solvent on the other, D may be defined by

 $D = \frac{h}{A} \frac{Q}{tC_1} \tag{20}$

where h is the effective distance through which the solute diffuses, A is the effective area of the membrane pores, Q is the amount which diffuses in time t, and C_1 is the concentration of the solution.

In equation (20) the value of the terms h/A differs for each membrane which is used, but is a constant for the same membrane regardless of the material which diffuses through it. It may therefore be considered a specific property of a membrane and termed the membrane constant. The membrane constant, designated by the symbol K, may be represented by the equation,

$$K = \frac{h}{A} = \frac{Dt}{Qml} \tag{21}$$

where Qml is the number of ml. of the initial solution that contains the amount of substance which has diffused through the membrane in time t. Since the diffusion constants for many simple substances are known, they can be used to evaluate K. This is done by trial experiments with substances of known diffusion constants. With the aid of equation (21), K is calculated from the diffusion data.

Using this method on carbon monoxide hemoglobin, Northrop and Anson (48) obtained a value of 2.73×10^{-7} cm. for r, and 68,500 for M. The latter value checks well with the values obtained by Adair and others for this protein. Since the initial work of Northrop and Anson, diffusion by the same procedure has been studied by McBain, Dawson and Barker (50) and by Mehl and Schmidt (51). The former workers estimated the diffusion of egg albumin under various conditions. A pronounced minimum of dif-

fusion was observed in the neighborhood of the isoelectric point. From the diffusion coefficient in this pH region, the molecular weight of egg albumin was calculated as 34,000±500. In a study of the diffusion of amino acids, Mehl and Schmidt arrived at the conclusion that the diffusion is not proportional to the molecular weight but rather to the molecular volume. Other theoretical aspects of the work of Mehl and Schmidt on diffusion are treated in Chapter XII.

A somewhat more complex method of studying the diffusion of proteins has been used by Svedberg (52) and by Tiselius and

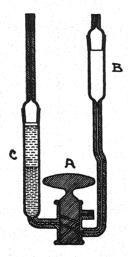


Fig. 15. Diffusion cell.

(Svedberg, T., Koll. Z. Ergänzungsband, 36, 53 (1925); Colloid Chemistry, 2nd Ed., New York (1928).

Gross (53). The apparatus which they used is illustrated in Fig. 15. In carrying out a determination a column of the protein solution is carefully superimposed over a column of the solvent. The diffusion of the protein is followed photographically, using ultraviolet light when necessary. An example of their work is illustrated in Fig. 16. This type of diffusion cell has to be mounted on a special suspension so as to be protected from the vibrations transmitted from the surroundings.

To evaluate the diffusion coefficient, D, from the measurements, recourse is had to the following procedure. The sharp boundary of contact which is formed when the protein solution is first superimposed over the solvent is photographed (see Fig. 16). This gives

the base line for calculating the rate of diffusion. After some time the boundary becomes somewhat blurred because of the diffusion. From the photographic changes, the concentration is evaluated at

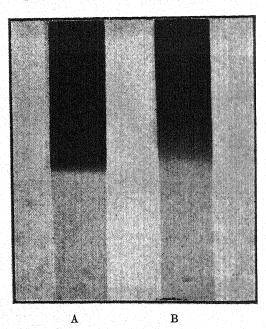


Fig. 16. Diffusion of alanine with the diffusion cell shown in Fig. 15. The movement of the boundary was determined by the fluorescence method.

(Svedberg, T., Koll. Z. Ergänzungsband, 36, 53 (1925).)

various distances from the initial boundary. The change in concentration resulting from diffusion conforms to the integral

$$C_x = \frac{C_0}{2} \left(1 - \frac{2}{\sqrt{\pi}} \int_0^y e^{-y^2} dy \right)$$
 (22)

In this equation C_x is the change in protein concentration at a distance x from the initial boundary in time, t, and C_0 is the initial concentration of protein. The term y is given by $y = (x/2\sqrt{Dt})$. Then by algebraic transformation,

$$D = \frac{x^2}{4y^2t} = \frac{1}{4y^2} \frac{x_1^2 - x_0^2}{t_1 - t_0}$$
 (23)

The term, $2/\sqrt{\pi} \int_0^{\nu} e^{-\nu^2} dy$, is the Gaussian probability integral which can be evaluated from statistical tables. By means of equation (23), D can be evaluated over the whole course of the diffusion change.

Some results obtained by Tiselius and Gross are given in Table XII. The authors concluded that the measured values of the diffusion coefficients with this apparatus are, in general, lower than the values of the coefficients calculated from their molecular weights and sedimentation velocities in the ultracentrifuge.

Diffusion alone, or in conjunction with mechanical force in the form of a superimposed pressure, may be used with membranes having much smaller pore size than that used by Northrop and Anson. In this case, it is not the rate of diffusion which is important, but the pore size of the filter. If the pores can be made uniform in size and the size graded at will, the proteins may be ar-

Table XII
Relation of Diffusion to Molecular Weight of Certain Proteins

Protein	Molecular weight by ultracentrifuge measurement	Diffusion coefficient, D, against H ₂ O at 20°
		cm.2/sec.×107
R-Phycoerythrin	209,000	4.00
R-Phycocyan	206,000	4.05
Carbon monoxide hemoglobin	68,000	6.30
Hemocyanin Helix Pomatia		1.05
Ovalbumin	34,500	7.70

(Tiselius, A., and Gross, D., Kolloid. Z., 66, 11 (1934).)

ranged in a series according to which membrane just allows them to pass or which one just keeps them from passing. Thus, comparative sizes of the molecules are obtained. Moreover, if the pores can be calibrated by means of filtering materials, such as colloidal particles of uniform and measured sizes, or by other means, a rough idea of the absolute size of the protein molecule may be obtained. Such methods are rarely very trustworthy because it is practically impossible to obtain uniform pore size or to calibrate the pores accurately.

(2) Molecular Weights from Measurements with the Ultracentrifuge. (a) The Ultracentrifuge. In addition to thermal forces, every molecule is subject to the influence of gravitational force, which, if it be large in comparison with the thermal forces causing diffusion, will cause molecules or particles to sediment. While protein molecules are not sufficiently large to settle under the influence of gravitational force, they can be made to sediment by the appli-

cation of a strong centrifugal field which acts in the same manner but with a very much greater force. This is the basis of the most important physical method which has been applied to the problem of molecular weights of proteins, namely, ultracentrifugation. This method, which has been extensively used, is due to the efforts of Svedberg and his co-workers (54, 55, 56). In order to cause proteins in solution to sediment at a reasonably rapid rate, it was found necessary to apply a centrifugal force of a much greater magnitude than that obtainable with any ordinary centrifuge. For this purpose the ultracentrifuge was constructed. Within certain mechanical and technical limits, the centrifugal force applied can be made as great as necessary, and can be carefully controlled as well. Svedberg has constructed two types of ultracentrifuge, one for speeds which will produce centrifugal fields from 50 to 8,500 times the force of gravity, the other for centrifugal forces of 8,000 to 400,000 times the force of gravity. The maximum speed of the latter is 80,000 r.p.m.4

(b) Sedimentation Equilibrium Method. The lower speed ultracentrifuge which produces a state of sedimentation equilibrium, as described by Svedberg and Rinde (58), allows the determination of particles that cannot be made visible in the ultramicroscope. It was designed upon the self balancing principle used in cream separators. A diagram of the working parts and a photograph of the rotor are given in Figs. 17 and 18. The centrifuge is driven by an electric motor through special couplings and gears. The rotor is surrounded by hydrogen at atmospheric pressure to eliminate frictional heating. The casing within which it rotates is immersed in a water thermostat.

The solution to be studied is enclosed in a small cylindrical cell with quartz windows. This is fitted into the rotor which has holes

⁴ The air driven spinning top type of ultracentrifuge appears to offer even greater potential possibilities for high velocities and greater centrifugal forces. However, the problem of eliminating certain types of instability inherent in this device has so far not been completely solved. These include precession, vertical vibration, and horizontal wobble. The present stage of development of this ultracentrifuge has been described by McBain and O'Sullivan (57). According to these authors, it should be possible to study the sedimentation not only of proteins, but also of simple organic and inorganic molecules when the apparatus is once perfected. A great boon in connection with the spinning top ultracentrifuge is that its comparatively low price will make it widely available.

Wyckoff and coworkers (81) have developed an air driven ultracentrifuge for molecular sedimentation. They have used it for the study of filtrable viruses and crystalline proteins. It can be constructed for much less cost than the one used by Svedberg.

to accommodate four cells. Fig. 17 shows several of the cells in place. To permit the solution to be observed and photographed

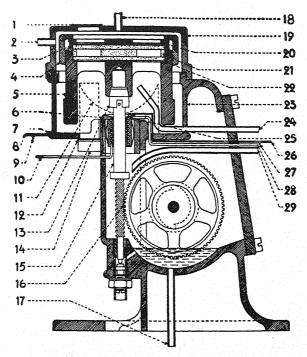


Fig. 17. Diagram of an ultracentrifuge for low and medium centrifugal forces. (Svedberg, T., and Rinde, H., J. Amer. Chem. Soc., 46, 2678 (1924).)

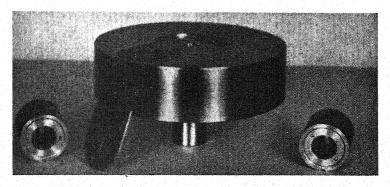


Fig. 18. The rotor of the low speed ultracentrifuge. (Svedberg, T., Chem. Rev., 14, 1 (1934).)

during centrifuging, the casing is provided with two quartz windows, each of which is fitted with an electromagnetic shutter.

The equation for the equilibrium state of centrifugation with this

apparatus can be developed either from kinetic or thermodynamic considerations. In accordance with the kinetic method, when a solution is centrifuged in a closed cell for a sufficient length of time, a state of equilibrium is reached between sedimentation and diffusion. At equilibrium the quantity ds of solute which is driven in time dt by the centrifugal force through a unit surface in the direction of the periphery is the same as that wandering in the opposite direction towards the center of rotation by virtue of diffusion.

The differential equation for sedimentation is

$$ds = c\omega^2 M (1 - V\rho) \frac{1}{F} dt \tag{24}$$

and for diffusion

$$ds = -RT \frac{dc}{dx} \frac{1}{F} dt \tag{25}$$

In these equations R is the gas constant, M the molecular weight of the solute, T is absolute temperature, F is the frictional force exerted upon a gram mole of solute, ω is the angular momentum of the centrifuge, V is the partial specific volume of the solute, ρ is the density of the solvent, c is the concentration of the solute, x is the distance from the center of rotation, and ds is the quantity of solute driven through a unit surface in time dt, either by centrifugal or thermal force. On equating (24) with equation (25), and rearranging algebraically, there is obtained

$$\frac{dc}{c} = -\frac{M(1 - V\rho)\omega^2 x dx}{RT} \tag{26}$$

Integrating this equation between two points, x_1 and x_2 , from the center of rotation yields

$$M = \frac{2RT \ln c_1/c_2}{\omega^2 (1 - V\rho)(x_1 - x_2)(x_1 + x_2)}$$
 (27)

In the sedimentation equilibrium method a gradient of increasing concentration of protein is established from the center of rotation outward toward the periphery. To estimate the molecular weight it is necessary (a) to determine the concentrations at several points situated x_1 and x_2 cm. from the center of rotation, (b) to know the temperature, (c) to know the speed of the centrifuge, and (d) to know the partial specific volume of the solute and the density of the solvent. If a substance of homomolecular composition is being investigated, the distribution of the gradient will be

such that the calculated molecular weight will be a constant at all distances from the center of rotation. The equation developed here is valid only for dilute solutions of an undissociated solute. With more concentrated solutions or with proteins somewhat removed from the isoelectric point where they are considerably dissociated, a correction must be applied for the altered activity coefficient of the protein. In most cases the proteins have been investigated in

TABLE XIII

The Observed Molecular Weights of Unelectrodialyzed Egg Albumin in the Sedimentation Equilibrium Centrifuge

UNELECTRODIALYZED EGG ALBUMIN

Concentration, 0.78 gm. per 100 cc.; conductivity, 7×10^{-5} mhos. in a 1.56 per cent solution at 17°; V = 0.749; $T = 288^{\circ}$; ρ , the density of the solution, 1.0004; b, the distance of outer end of solution from axis of rotation, 4.73 cm.; length of column of solution, 0.54 cm.; thickness of column, 0.200 cm.; $x_2 - x_1$ interval, 0.0496 cm.; speed, 10,550 r.p.m. ($\omega = 351.7 \pi$); aperture of the quartz lens, f40; the number of exposures was 3 in every case except the first where it was 6.

Distance	s, cm.	Mean co gm. per l			Calcd. values for a mixture 94 per cent of 34,000+6 per cent of 170,000
x_2	x_1	C ₂	c_1	M (obs.)	
4.680	4.630	1.0455	0.9149	45,300	47,600
4.630	4.581	0.9149	0.8077	42,750	43,700
4.581	4.531	0.8077	0.7128	43,300	40,400
4.531	4.482	0.7128	0.6386	38,500	38,000
4.482	4.432	0.6386	0.5754	36,950	37,800
4.432	4.382	0.5754	0.5196	36,550	36,300
4.382	4.333	0.5196	0.4708	35,700	35,450
4.333	4.283	0.4708	0.4277	35,200	35,100
4.283	4.233	0.4277	0.3890	35,150	35,000

(Svedberg, T., and Nichols, J. B., J. Amer. Chem. Soc., 48, 3081 (1926).)

dilute solutions and at the isoelectric point where the formula is sufficiently exact. In cases where the protein is not soluble at the isoelectric point it is of course necessary to dissolve it in whatever manner that is feasible, even if it is no longer isoelectric, and then to apply proper corrections to the results. Since the process has to do with an equilibrium, the time and the frictional force do not enter into equation (27). Therefore this method of determining molecular weights is theoretically quite sound, being as free from assumptions as any of the methods which are based on the colligative properties of solutions. Since the equation was derived from

the condition of sedimentation equilibrium, it is valid only when an equilibrium has been reached.

The presence of more than one molecular species can be detected by the sedimentation equilibrium method. Under a given centrifugal force, the larger molecules are concentrated more toward the periphery. Consequently the calculation of the molecular weight will yield values which increase the greater the distance from the axis of rotation. This is illustrated by the data given in Table XIII, showing the distribution of the apparent molecular weight in

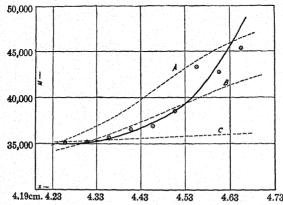


Fig. 19. Relation between molecular weight and distance. Full line: calculated curve for a mixture of molecules, 94% of 34,000 and 6% of 170,000. Circles: experimental values from Table XIII. Dotted lines: hypothetical mixtures of molecules. A. 40% of 17,000, 10% of 34,000, 10% of 51,000 and 40% of 68,000. B. 30% of 17,000, 30% of 34,000, 20% of 51,000 and 20% of 68,000. C. 95% of 34,000 and 5% of 68,000.

(Svedberg, T., and Nichols, J. B., J. Amer. Chem. Soc., 48, 3081 (1926).)

an unelectrodialyzed specimen of egg albumin. To estimate the number of components and the exact values of their molecular weights in such a mixture is a difficult and somewhat uncertain procedure. To do this one makes various assumptions regarding the number and size of the molecules present and from the assumed values proceeds to calculate the expected concentration gradient. The calculated gradient is then compared with the one experimentally observed. The results of such a series of calculations on the unelectrodialyzed egg albumin of Table XIII are shown in Fig. 19.

(c) Sedimentation Velocity Method. While theoretically quite sound, certain experimental difficulties are encountered in the sedimentation equilibrium method. Attainment of equilibrium is not rapid, usually requiring several days. In this length of time certain

of the proteins may become appreciably altered. To overcome this defect, the high speed centrifuge, by which measurements can be completed in a few hours, was devised. In the later method the sedimentation velocity is increased to such a magnitude that the velocity of diffusion due to thermal agitation becomes negligible in comparison. To accomplish this a very high centrifugal force is required. Besides the shorter time required, another advantage of the sedimentation velocity method is that it is easier to determine with it the nature of a mixture of molecules when they are present in the solution.

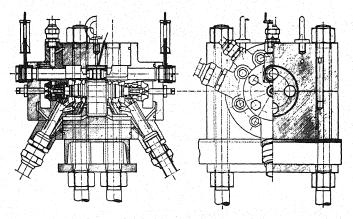


Fig. 20. Diagram of the oil turbine ultracentrifuge. (Svedberg, T., and Nichols, J. B., J. Amer. Chem. Soc., 49, 2920 (1927); Svedberg, T., Colloid Chemistry, New York, 1928, p. 157.)

The design of the first oil turbine centrifuge (59) and its rotor are shown in Figs. 20 and 21. Fig. 21 also shows the type of cell used with the high speed rotor. These cells are of very heavy quartz plates so cut and fitted that a small compartment is open in the center. In this is placed the solution which is being studied. In order to avoid heating due to the excessive speeds, all bearings have forced oiling. Watercooled oil is circulated around the moving rotor shaft, while the open area around the rotor itself is occupied by hydrogen gas at a pressure of about 15 mm. of mercury. Thermocouples are used to measure the temperatures of different portions of the apparatus. It was found that the cooling was quite efficient, since a temperature difference of only 1.5° exists between the rotor and casings at the maximum speed of 42,000 r.p.m.

An improved oil turbine type of ultracentrifuge has been developed by Svedberg (55). It is capable of attaining speeds ranging

between 5,000 to 80,000 r.p.m. The rotor of this new ultracentrifuge is pictured in Fig. 22. It is made of chromium-nickel steel and is oval in shape in order to lessen the strain. This rotor was tested

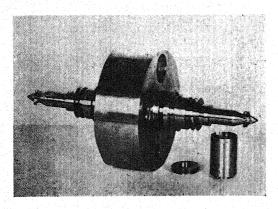


Fig. 21. The rotor of the oil turbine centrifuge.

(Svedberg, T., and Nichols, J. B., J. Amer. Chem. Soc., 49, 2920 (1927); Svedberg, T., Colloid Chemistry, New York, 1928, p. 155.)

at 78,000 and has been run regularly at 75,000 r.p.m. The latter velocity corresponds to a centrifugal field of 400,000 times gravity at the center of the cell. The housing and installation are shown in

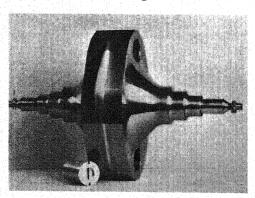


Fig. 22. Rotor of the improved oil turbine ultracentrifuge. (Svedberg, T., Chem. Rev., 14, 1 (1934).)

Fig. 23. In this figure there is also a photograph of a rotor which burst at 58,000 r.p.m.

The rate of sedimentation of the protein under the influence of the high centrifugal force is followed photographically while the rotor is in motion. To accomplish this, quartz windows are placed in the casing of the ultracentrifuge so that the quartz cells pass the windows during each revolution. The optical system and the camera are placed in line with the windows. Visible light is used with colored proteins such as hemoglobin, and ultraviolet light with solutions of colorless proteins. The light is passed through heat and color filters and rendered parallel before being passed through the cell. By this photographic method, a series of pictures is obtained which represents the different stages in the sedimentation of the protein under investigation.

The speed of the ultracentrifuge is determined by the use of a stroboscopic tachometer mounted behind the camera. This instrument consists of a disc which can be rotated at varying velocities with a slot cut in it at some point. On sighting at a marked point on the revolving rotor through the slot in the stroboscopic disc, the point will appear to be standing still when the rotating velocities of the rotor and the stroboscopic disc are the same. At this point the measured velocity of the stroboscope which is recorded by an electrical counting device represents the rotational speed of the ultracentrifuge.

In summarizing it may be stated that the turbine ultracentrifuge consists of the following essential units: (a) the centrifuge, (b) the oil circulating system, (c) the vacuum and hydrogen gas system, (d) the optical set-up for obtaining photographs, and (e) the stroboscopic tachometer for measuring the velocity of rotation.

The following is the theory of the sedimentation velocity method as reported by Svedberg and Nichols (59). After the short initial period, the centrifugal force per gram mole, which is $M(1-V\rho)\omega^2 x$, becomes equal but opposite in sign to the frictional force, f(dx/dt), where f = (RT/D) for dilute solutions. D is the diffusion constant. The other symbols have the same meaning as given in the development of the equation for sedimentation equilibrium. On equating and solving for M, there is obtained

$$M = \frac{RT}{D(1 - V\rho)} \frac{dx/dt}{\omega^2 x} \tag{28}$$

If the specific sedimentation velocity, s, which is a constant for every molecular species at a given temperature and for a given solvent, is defined as

$$s = (1/\omega^2 x) dx/dt \tag{29}$$

then

$$M = \frac{RTs}{D(1 - V\rho)} \tag{30}$$

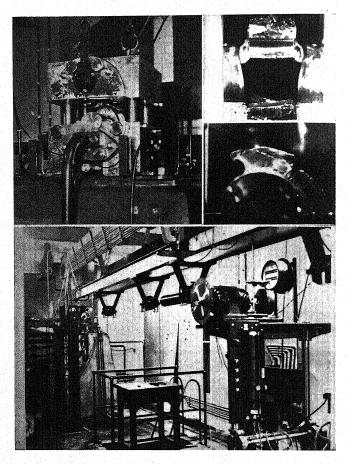


Fig. 23. Housing and installation of the improved oil turbine ultracentrifuge. The upper right hand figure shows the remains of a rotor that burst at 58,000 r.p.m.

(Svedberg, T., Chem. Rev., 14, 1 (1934).)

For large intervals of x, the integrated form of equation (30) must be used:

$$M = \frac{RT \ln (x_2/x_1)}{D(1 - V\rho)\omega^2(t_2 - t_1)}$$
(31)

In using this equation, it is necessary to know D, the diffusion constant. This may be calculated from the rate of sedimentation in the ultracentrifuge by the use of equations (22) and (23) which have been developed in the section on diffusion. For more details concerning the method and the interpretation of the data, the original articles should be consulted.

As is readily seen, the method of obtaining and calculating the data by the sedimentation velocity method differs radically from that of the sedimentation equilibrium method. While less sound theoretically, it possesses the important advantage of greatly shortening the time required for an experiment.

In the sedimentation velocity method, the solute moves away from the center of rotation leaving a boundary between the protein solution and the clear solvent. The movement of this boundary is photographed from time to time to obtain the data required for the molecular weight calculation. The photograph obtained in such

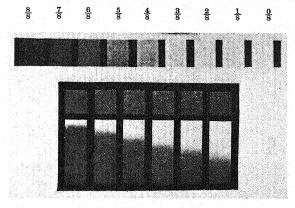


Fig. 24. The sedimentation of carbon monoxide hemoglobin in the oil turbine ultracentrifuge. The top strip gives the concentration scale.

(Svedberg, T., and Nichols, J. B., J. Amer. Chem. Soc., 49, 2920 (1927); Svedberg, T., Colloid Chemistry, New York, 1928, p. 161.)

an experiment is shown in Fig. 24. With a sufficiently strong centrifugal field of force it would be possible to measure directly the movement of the boundary of the dissolved substance with time. Actually the boundaries are usually not sharp on account of the blurring caused by diffusion. The true position of the boundary is calculated from the theory of diffusion provided a region of unchanged concentration is still present. From the theory of diffusion it follows that the true boundary is the surface where the concentration is half of that of the original concentration of the solution. The variation in protein concentration at different points in the ultracentrifuge cell is estimated from the varying intensity of color of different portions of the photograph of the sedimenting protein. The intensity differences can be measured with a photometer and the corresponding concentrations plotted. From the curve so ob-

tained, the position of the 50 per cent relative concentration, which represents the theoretical boundary, is read.

The sedimentation velocity method also allows the easy detection of the presence of a mixture of different size molecules which, because of their different rates of sedimentation, give a stepwise appearance to the photographs. On account of the different sedimentary velocities, a partial separation of the particles of different sizes takes place, as it also does in the sedimentation equilibrium

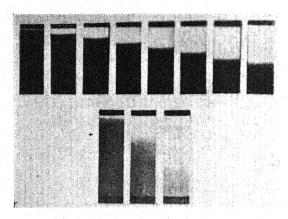


Fig. 25. Sedimentation in the ultracentrifuge of a monodisperse sol (hemocyanin) and a polydisperse sol (gold). The top row shows the sharp boundary obtained in the sedimentation of the homomolecular hemocyanin. The bottom row shows the very diffuse boundary obtained with a gold sol which is polymolecular.

(Svedberg, T., and Chirnoaga, E., J. Amer. Chem. Soc., 50, 1399 (1928); Svedberg, T., Colloid Chemistry, New York, 1928, p. 166.)

method. In such cases the boundaries become much more diffuse than would correspond to diffusion only.

The uniformity or heterogeneity of the particle size can be best tested if a strong centrifugal field is employed, since then the velocity of sedimentation is so fast that the time for diffusion which causes blurring is greatly reduced, and, with a homomolecular substance, a quite sharp boundary is obtained. If blurring occurs under these conditions, it must be due to the presence of a mixture of different sized particles. An illustration of the difference which may be observed in the sedimenting boundaries is shown in Fig. 25. The figure shows that hemocyanin, which is homomolecular, has a very sharp boundary, while the boundary zone of colloidal gold, which is polydisperse, is extremely diffuse.

In Fig. 26 is shown another interesting illustration of the difference in the sedimentation rates of proteins of different sizes. At pH 6.5, two components, one of low and the other of high molecular weight, apparently in equilibrium with each other, are present in octopus hemocyanin. Because of the great difference in the sedi-

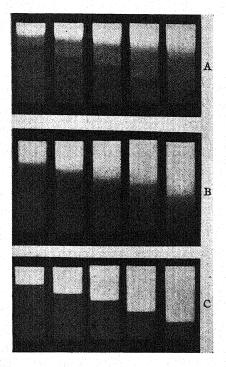


Fig. 26. Sedimentation of octopus hemocyanin in the ultracentrifuge at varying values of pH. Section A shows the difference in the rate of sedimentation of the two hemocyanin components present in the solution at pH 6.5. Section B is a photograph of the sedimentation at pH 7.3, where the lower molecular weight component preponderates. Section C represents the sedimentation rate at pH 8.0, where the high molecular weight hemocyanin preponderates.

(Svedberg, T., and Eriksson, I-B., J. Amer. Chem. Soc. 54, 4730 (1932).)

mentation rates of the two components, two boundaries representative of each component occur in the photograph (Fig. 26 A). At pH 7.3 (Fig. 26 B), only one boundary, corresponding to the low molecular weight component, and in Fig. 26 C the single boundary of the high molecular weight component are visible. Owing to the slower diffusion and probably also to a higher degree of homogeneity, the boundary of the high molecular component is

much sharper than that of the low molecular component. The changes in the composition of the protein with respect to pH are reversible.

Each of the components of a polymolecular mixture can be characterized by evaluating its sedimentation constant. Reduced to water as a solvent, the sedimentation constant at 20° is given by the relation

$$S_{20}^{\circ} = dx/dt \cdot \frac{\eta(1 - V\rho_{o})}{\eta_{0}\omega^{2}x(1 - V\rho)}$$

$$(32)$$

In this equation dx/dt is the observed sedimentation velocity, ω is the angular momentum, x is the distance from the center of rotation, η is the viscosity of the solution, η_0 is the viscosity of water at 20°, V is the partial specific volume of the solute, ρ is the density of the solution, and ρ_0 is the density of water at 20°.

Whether or not a protein is spherical and the degree it deviates from the spherical shape can be estimated by calculating the dissymmetry number. This is given by the ratio

$$\frac{f}{f_0} = \frac{\frac{M(1 - V\rho)}{S}}{6\pi\eta N \left(\frac{3}{4} \frac{MV}{\pi N}\right)^{1/3}}$$
(33)

In this equation the numerator, f, is the measured molar frictional coefficient and the denominator, f_0 , is the theoretical molar frictional coefficient of a spherical molecule of the same molecular weight, M, and specific volume, V. Of the other terms in equation (33), S is the specific sedimentation velocity, ρ is the density of the solution, η is the viscosity of the solution, and N is Avagadro's number. When a molecule is spherical its dissymmetry number is equal to unity. As a molecule deviates from the spherical shape, its dissymmetry number becomes increasingly greater than unity.

The summary of the values of the molecular weights of the various types of proteins, compiled by Svedberg and Eriksson-Quensel (60), is given in Table XIV. Other pertinent data included in the table are the pH regions of stability, the sedimentation constants, the diffusion coefficients, and the dissymmetry numbers of the proteins.

The molecular weights of the chief components of certain protein systems of a polymolecular nature are given in Table XV.

The work of Svedberg and his associates in this field has become

Molecular Weights and Molecular Constants of Important Types of Proteins as Determined by the Ultracentrifuge TABLE XIV

								Dissvm-
Protein	Source	pH region of stability	Sedimen- Diffusion tation coeffi- constant cient S20 X 10 ¹³	Diffusion coefficient $D_{20} \times 10^7$	M_v^{1}	M_{E^1}	Units of 36,400	metry number $\frac{f}{f_0}$
Ervthroernorin	Lampetra blood		1.90	10.10	18,300	19,000	Hla	1.2
Erythrocruorin	Arca blood		3.46			33,600		0.1
Erythroeruorin	Chironomus blood		2.00			31,400		1.6
Lactoglobulin	Cow's milk	1.0 to 9.0	3.14	00.6	33,900	37,800		
Pepsin	Pig's stomach		3.30	00.6	35,500	39,200	-1	
Insulin	Pig's pancreas	4.5 to 7.0	3.47			35,100		1.0
Bence Jones protein	Human urine	3.5 to 7.5	3.55			35,000		1.0
Ovalbumin	Hen's egg	4.0 to 9.0	3.55	7.76	43,800	40,500		1.1
Hemoglobin	Horse blood	6.0 to 9.0	4.50	6.30	69,000	000'89		1.2
Hemoglobin	Human blood		4.50	6.90	63,000		2	1.2
Serum albumin	Horse blood	4.0 to 9.0	4.50	6.45	67,100	66,900		1.2
Serum globulin						1		1
(chief component)	Horse blood	4.0 to 9.0	7.10			150,000	4	
Phycoerythrin	Ceramium	4.4 to 8.0	12.00	4.00	290,000	292,000		1.2
Phycocyan	Ceramium	2.5 to 5.0	11.70	4.05	279,000		∞	1.2
Edestin	Hemp seed	3.5 to 9.7	12.80	4.00	303,000			1.2
Excelsin	Brazil nut	5.5 to 10.0	13.30	4.26	294,000			1.1
Amandin	Almond	4.3 to 10.0	12.50	3.62	329,000			1.3
Hemocyanin	Pandalus blood		17.40			397,000	$1 \times 400,000$	
Hemogyanin	Palinurus blood		16.40	3.40	446,000	460,000		I.3
Thyroglobulin	Pig's pancreas		19.20	2.39	000,969	675,000		1.5
Hemocyanin	Nephrop blood		24.50		766,000		$2 \times 400,000$	1.2

Table XIV—(Continued)

Protein	Source	pH region of stability	Sedimen- Diffusion tation coeffi- constant cient S ₂₀ × 10 ¹³ D ₂₀ × 10 ⁷	Diffusion coeffi- cient D ₂₀ ×107	M_v^1	M_{E}^{1}	Units of 36,400	Dissymmetry number f f f
Hemocyanin	Homarus blood		22.60	2.78	752,000	784,000		1.3
Erythrocruorin	Planoribis blood		33.70	1.96	1,634,000	1,539,000	$4 \times 400,000$	1.3
Hemocyanin	Calocaris blood		34.00			1,327,000		1.2
Hemogyanin	Octopus blood		49.30	1.65	2,785,000		$7 \times 400,000$	1.4
Hemocyanin	Eledone blood		49.10	1.64	2,791,000			1.4
Erythroeruorin	Arenicola blood	2.6 to 8.0	57.40			3,000,000	$8 \times 400,000$	1.3
Hemocyanin	Rossia blood		56.20	1.58	3,316,000			1.4
Erythroeruorin	Lumbricus blood	2.6 to 10.0	06.09	1.78	3,190,000	2,946,000		1.2
Hemocyanin	Helix blood		98.90	1.38	6,630,000	0,200,902,9	$16 \times 400,000$	1.3
Hemocyanin (chief								
component)	Buscyon blood		99.70	1.38	0,000,089,9		6	1.2
Hemocyanin	Buscyon blood		126.20	1.17	9,660,000		$ 24 \times 400,000$	

¹ M, represents the molecular weight from sedimentation velocity, and M_E that from sedimentation equilibrium measurements. (Svedberg, T., and Eriksson-Quensel, I.-B., Tabulae Biologicae Periodicae, V (4), 352 (1935-36).)

so extensive that space does not permit a full discussion of all of the results. From Tables XIV and XV it will be seen that numerous proteins from many sources have been investigated. Besides the ordinary proteins, the various respiratory pigments found in nature have been especially studied. Of particular interest are the hormone proteins (insulin and thyroglobulin), pepsin, the recently isolated

Table XV

Molecular Weight Distribution of Certain Polymolecular Proteins as

Determined by the Ultracentrifuge

Protein	Source	Sedimentation constant $S_{20} \times 10^{13}$	Molecular weight of chief molecular components	Refer- ence*
Lactalbumin (purified) Gelatin		2.0 to 3.0	10,000 to 100,000	(61) (62, 63)
Casein	Cow's milk	3.0 to 6.0	1	(64)
Casein (Hammersten). Zein	Corn	13.6 1.5 to 3.5	375,000 60 per cent of 35,000	(64) (65)
Myoglobin	Muscle	2.0	16,000)	(00)
Hyogrosiii	Muscle	4.0	34,000 68,000	(66)
Cocosin	Coconut	11.37	104,000) 208,000)	(67)
Pomelin	Orange seed	2.2	17,000)	
		11.38	210,000}	(68)
		16.75	320,000	
Euglobulin	Blood serum		Complex mixture	(69)
Pseudoglobulin Warburg's yellow oxi-			50,000 to 100,000	(69)
dation enzyme			28,000 to 95,000	(70)
Mosaic virus protein	Tobacco plant		15 to 20 million	(71)

^{*} The references refer to the list at the end of the chapter.

yellow oxidation enzyme, and the virus protein of the tobacco mosaic disease.

Besides the proteins listed in Tables XIV and XV, attention has been devoted to such protein-containing systems as blood (72, 73), milk (74), and wheat flour (75). The work in this field has not found ready interpretation. According to McFarlane (73), the protein components isolated by salting out blood serum do not find their counterpart in the whole serum. Wheat flour seems to be a complex mixture of proteins which at present defies interpretation. In milk, casein is present only as a coarse polydisperse suspension. The milk

whey or milk serum was found to contain at least three different proteins. These, apparently, are a lactalbumin and two kinds of lactoglobulin. Also worthy of note is the attempt to estimate the molecular size of antibodies from the globulin fraction of the serum proteins (76).

(d) Units of Molecular Weight. As a result of very extensive investigations, Svedberg (77) has announced a surprising relationship governing the values of molecular weights. The molecular weight values obtained on the proteins which he and his coworkers have investigated, with the possible exception of those whose molecular weights are in the millions, are all stated to be in the immediate neighborhood of 36,400 or some simple multiple of this value. The unit value first suggested was 34,500, but has recently been increased to 36,400 (60). As is shown in Table XIV, the multiple appears to be 2, 4, or 8 times the unit weight of the simpler proteins. Proteins of a higher molecular weight appear to have values approximating simple multiples of 400,000. If this relationship of Svedberg's can be confirmed by other methods of investigation, it will probably prove to be one of the most fundamental generalizations bearing on our understanding of the way in which protein molecules are built up. However, the evidence for the existence of proteins with a molecular weight of half the unit value of 36,400 (e.g., myoglobin and Lampetra erythrocruorin), makes it difficult to maintain that the proteins are built up in accordance with Svedberg's hypothesis. Because these particular proteins with molecular weights less than the unit value have been found only in systems also containing proteins of a high molecular weight, it has been suggested that they may be secondary degradation products.

In this connection it should be stated that in most cases the proteins were found to have a certain range of stability with respect to pH (see Table XIV). Outside of this stability range, the molecular weights are altered by aggregation or disaggregation, usually the latter. In some cases this alteration in molecular weight takes place in terms of units of 36,400. In other cases, as, for example, with gelatin, units as low or lower than 10,000 were detected. It would seem somewhat difficult under the circumstances to distinguish between an actual rupture of the protein molecule and a simple aggregation or disaggregation of the units of structure. Thus, if the Svedberg unit were itself made up of still smaller units, it would be difficult to state that these small units were not held together by the same forces holding the larger units together.

Many other discrepancies might be pointed out, and the sum total of these is sufficient to cause us to consider that Svedberg's generalization is still unproven, at least until further confirmation is obtained and the conflicting data are explained. Vickery (78) has pointed out that the unit of 36,400 is, in various respects, much less obvious than a unit of half this size. Such an assumption would render easier the interpretation of the ultracentrifugal study of milk proteins as well as to bring some of the conflicting data mentioned above into line with the unit hypothesis of protein structure.

Pedersen (79) has reported certain observations which may prove to have an important bearing on the relation of the molecular size of proteins to each other in a polymolecular protein mixture. He noted that the addition of a protein or a polypeptide to a solution of a second protein appeared, from the sedimentation velocity data, to produce a disaggregation of the protein. Thus the addition of clupein to serum albumin or hemoglobin apparently resulted in the appearance of some protein of considerably lower molecular weight than the parent albumin or hemoglobin. The cause of this phenomenon has not been established.

The generalization of Svedberg is one which merits further study and elucidation. If it, or some modification of it, can be confirmed, and the discrepancies with other means of investigation eliminated, it should go very far toward establishing a sound basis for our ideas on protein structure.

6. MOLECULAR WEIGHTS FROM SURFACE TENSION MEASUREMENTS

An ingenious method for estimating molecular weights of proteins using surface tension measurements has been developed by du Noüy (83). When a material in solution is of such a nature that it concentrates on the surface, it lowers the surface tension. Among such materials are soaps, bile salts, and proteins. The maximum lowering of surface tension occurs when the material has formed a uniform film on the surface. Ordinarily this film is only one molecule thick though it may be more. By altering the concentration of the solute some particular dilutions will be found in which these surface films will be composed of molecules having a definite orientation. Since the molecule is three different positions, different concentrations will give films with a different molecular orientation. This fact serves as a basis for du Noüy's method. Protein solutions are diluted from 1/10,000 to 1/1,000,000 in successive steps. The

static surface tension is measured with the du Nouy tensiometer. It consists essentially of a small horizontal platinum ring which is dipped into the solution and then pulled loose by gradual increase of stress produced by torsion in the support. The force which is necessary to remove the ring can be measured quite accurately and from this the surface is calculated. When a series of dilutions of protein solutions are so measured, the curve obtained by plotting the surface tension against the dilution shows a number of minima. At least three of these should correspond to certain positions of orientation of the molecule and these three minima should show the greatest frequency in a series of measurements. For purposes of obtaining molecular weight values from such data, three general assumptions are made. These are: (a) in very high dilutions, all of the protein in the solution will collect in the liquid-air and liquidglass interfaces so that the thickness of the surface film may be calculated from the formula, $L = MC/S\delta$, where L is the thickness, C is the concentration, M is the mass of the mixture in the watch glass, S is the area of the adsorbing surface, i.e., the total surface of the liquid in contact with air and glass, and δ is the specific gravity of the substance in solution; (b) the molecules have some regular shape, the details of which must be empirically assumed, e.g., that the molecule is a prism with some particular shape of base. and that no interpenetration of molecules takes place; (c) that the specific gravity of the surface film may be taken as some definite known value, e.g., that of the dry protein, thus neglecting the unknown influence of hydration and other factors.

If these three assumptions are granted, then the thickness of the film at any definite dilution may be calculated from the known mass of protein, the known surface, and the assumed specific gravity. The thickness of the film at each of the three important minima should represent a dimension of the molecule if the film is assumed to be monomolecular. This gives the three dimensions of the protein molecule. If, for example, its shape is assumed to be a parallelopiped, the product of the three dimensions gives the volume occupied by one molecule. The volume per molecule multiplied by the specific gravity of the protein and by Avogadro's number gives the molecular weight.

On the basis of this method, du Noüy (84) obtained values ranging from 23,500 to 30,800 for egg albumin, depending on the shape assumed for the molecule. He favors the higher value as the more probable partly because it comes nearest to checking with the val-

ues obtained by other methods. De Caro (85), using the du Noüy technique, has determined the molecular weight of muscle protein as 3,200,000, and from this he has proposed a mode of muscle contraction and a theory of the structure of muscle fiber. De Caro and Laporta (86) have studied the variation of surface tension with pH in solutions of serum albumin, gelatin, and egg albumin. They propose the value of 66,167 for the molecular weight of serum albumin. Laporta (87) has studied hemoglobin by the same method and obtained a value of 68,700 for the molecular weight of this protein. Herčik (88) has summarized and discussed the various values of protein molecular weights obtained in this manner but has added no values of his own.

The use of the surface tension method is favored on account of its ease and rapidity, as well as the fact that the protein need not be rendered isoelectric, or even purified. Whether values obtained in this manner are to be considered as trustworthy appears doubtful. Even if the interpretation of the minima in the surface tension curve is exact, which may be questioned, the other assumptions may very well be in considerable error. Large variations in the result will be introduced by different assumptions regarding the shape of the molecule. In all of the cases studied, other values are available, and these could be used to check the assumptions made. It would be interesting to know what these assumptions would have been, and what values would have been obtained had there been no other data at hand with which to compare the results.

7. THE ASSOCIATION OF AMINO ACIDS AND PROTEINS WHEN IN SOLUTION

The evidence for the association of amino acids and proteins, when in aqueous solution, is in a controversial state as is evidenced by the following brief résumé of the literature.

Measurement of the colligative properties of amino acid and protein solutions offers one line of evidence bearing upon this problem. If association of amino acid or protein molecules occurs in aqueous solutions, the depression of the freezing point and the osmotic pressure of such solutions should be less than would be expected from the formula weight concentration. However, this type of evidence needs to be treated with caution, since factors other than association may possibly lead to smaller values of the freezing point depression or the osmotic pressure than would theoretically be expected.

Association of amino acids should have an influence on the dielectric constant of the solution. This should be reflected by abnormal deviations in a curve which shows the relationship between concentration and the dielectric constant. It is further to be expected that such deviations from ideal behavior will be greater in concentrated than in dilute solutions.

The data of Adair (25) on the change of the osmotic pressure of hemoglobin with concentration of the protein show a positive deviation from the curve for an ideal solution. A similar deviation was noted by Burk and Greenberg (29) and Burk (43) in the case of proteins which were dissolved in aqueous urea solutions. The positive deviation from a linear curve can be attributed to deviation from the laws which govern ideal solutions. The positive deviation can also be taken as evidence against association of the protein molecules which were used in these experiments.

Deviation of the osmotic pressure in a direction which would indicate an association of the protein was observed with the globins from various animal species by Roche, Roche, Adair and Adair (41). Frankel (89) determined the osmotic pressures of aqueous solutions of gelatin at 6° and at 30°. The molecular weight of this protein, calculated from the data obtained at 6°, was about 54,000, while that calculated from the data at 30° was about 20,000. It is to be expected that a greater amount of aggregation of the gelatin molecules occurs at the lower than at the higher temperature. This idea is not supported by the work of Kunitz (90). He found that the osmotic pressure-concentration curve for aqueous solutions of isoelectric gelatin at 35° deviates in a positive direction from linearity.

Freezing point data on aqueous solutions of glycine, particularly those which have been reported by Lewis (91), Frankel (92), and Scatchard and Prentiss (93), indicate that the depression of the freezing point of water caused by this amino acid is less than would be expected. Lewis assumed that some of the glycine is associated. He calculated the constant for the reaction

$$K = \frac{(\text{Glycine})^2}{(\text{Glycine})_2}$$

to be 8.4. Similar calculations, using the data of Frankel and Scatchard and Prentiss, give 7.0 and 7.7, respectively. According to Lewis (91), about 10 per cent of the glycine molecules which are present in a 1 molal solution are associated. In the case of alanine, Frankel (92, 94) found no abnormality in the depression of the

freezing point of solutions over a range in concentration from 0.269 to 1.68 molal. The indices of refraction of his alanine solutions are directly proportional to the concentration of the solute. Over a concentration range of 0.107 to 1.59 molal the indices of refraction of glycine solutions were less than would be expected if there was no aggregation of the molecules. However, Craig and Schmidt (95) have pointed out that such an effect may be due to hydration.

Hoskins, Randall and Schmidt (96) investigated the freezing points of aqueous solutions of aspartic and glutamic acids and their monosodium salts. The data were interpreted as indicating that the undissociated parts of these amino acids in aqueous solutions exist to a considerable extent as neutral aggregates, while the ionized parts of the monosodium salts of these amino acids are probably micellated to a slight extent.

Measurements of the dielectric constants of aqueous solutions of glycine and alanine by Hedestrand (97), Devoto (98), and Wyman and McMeekin (99) show that the dielectric constants increase linearly with the concentration of the amino acid. Data obtained in this laboratory by Lindquist and Schmidt (82) indicate a similar relationship. Their work was carried out on aqueous solutions of glycine, alanine, proline, and hydroxyproline. They consider that if aggregation of these amino acids occurs, the amount is less than 5 per cent. On the other hand, Frankenthal's (100) data for glycine and alanine solutions show that as the concentrations of these amino acids were increased, the increase in the dielectre constants per mole of amino acid was less than that demanded by a linear relationship.

The work of Fuoss and Kraus which has been summarized by Kraus (101) seems to offer a likely explanation for the apparent absence of association of the zwitterions in the solutions which have been studied. They considered the work which is necessary to separate two elementary charges on the assumption that the only forces which are acting between the charges are Coulombic forces. In a solvent having a dielectric constant of 78, this being the dielectric constant of water at 25°, they calculate that the energy which is necessary to separate two spherical ions which are separated by a distance of 5 Å is 840 calories per mole. This is comparable to the mean translational energy of the molecules at ordinary temperatures. They further calculate that two ions with their charges separated by a distance of 6.4 Å will be completely dissociated in any medium having a dielectric constant greater than 40.

Although these calculations refer to the case of a binary electrolyte, it seems quite probable that the ideas can be applied to aqueous solutions of amino acids. In view of the fact that the values of the dielectric constants of amino acid solutions are considerably larger than the dielectric constant of water, the energy which is necessary to dissociate any possible molecular complex which might be formed, assuming that only Coulombic forces are acting, should be of the order of magnitude of the mean translational energy of the molecules at ordinary temperatures. On this basis association of the zwitterions would not be expected to occur in dilute aqueous solution.

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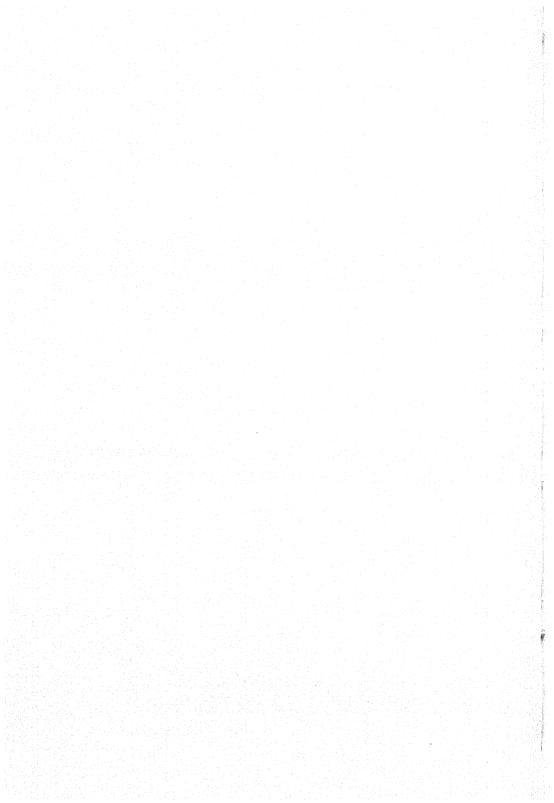
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CHAPTER IX

CERTAIN CHEMICAL AND PHYSICAL CHARACTERISTICS OF THE PROTEINS



CHAPTER IX

SECTION I. THE COAGULATION OF PROTEINS

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1. GENERAL DESCRIPTION OF COAGULATION

(1) Heat Coagulation. If almost any tissue is extracted with salt solution and the extract is boiled, a precipitate is formed which does not redissolve when the solution is cooled again. The insoluble substance is protein. The change in the protein from a soluble to an insoluble substance is known as heat coagulation. Not all proteins are heat coagulable (gelatin is not) but almost all of the ordinary tissue proteins are heat coagulable. Proteins can be separated from non-protein material by heat coagulation and two different proteins can be separated from each other by coagulation, when the conditions for their coagulation are very different. Heat coagulation therefore has been a fundamental procedure in preparative and analytical work with proteins from the very beginning of such work (1).

If a native protein is first dried and then heated to 100°, the protein retains its solubility. Water must be present (2, 3) in order that heat coagulation of protein can take place.

- (2) Solubility of Coagulated Proteins. Heat coagulated protein is insoluble at its isoelectric point. It is dissolved by the addition of sufficient acid, alkali, urea, or a salicylate, and it is reprecipitated when the added acid or alkali is neutralized or the added urea or salicylate is removed. The original or *native* protein is soluble in salt solution even at its isoelectric point.
- (3) The Two Steps of Coagulation (4, 5, 6, 7, 8). Protein which is heated in acid or alkaline solution does not undergo any visible change. When the heated solution is cooled and neutralized, however, the protein in the previously heated solution immediately precipitates. This experiment shows that ordinary heat coagulation consists of two entirely distinct reactions. The first reaction is the change of the protein into a form which is insoluble at the isoelectric point. This change, which is called denaturation, is caused by

heat and is characteristic of proteins. The second reaction is the precipitation of the heat denatured protein at its isoelectric point. This precipitation does not require heat and is similar to the precipitation of any other insoluble substance. When protein is heated in acid the two reactions are separated. The protein is denatured by the heat; the heat denatured protein, however, is kept in solution by the acid. Confusion has been caused by the fact that the word "coagulation" when applied to colloids refers, in general, merely to the precipitation of the colloid, whereas when the word "coagulation" is applied to proteins in particular it refers to the precipitation plus the preliminary chemical change which makes the precipitation possible.

- (4) Different Ways of Denaturation. A change of protein into a form which is insoluble at the isoelectric point, and which is similar to the change produced by heat, can also be produced by acid and alkali; alcohol and acetone; saturated neutral solutions of urea (9, 10, 11), sodium salicylate and potassium iodide; X-rays, ultraviolet light, and visible light plus a suitable photo-sensitizer (12, 13); shaking (14, 15), very high pressure (16); trichloroacetic, tungstic, and sulphosalicylic acids; and by other means. When secondary reactions can be avoided, denaturation by all of these agents produces similar changes in proteins. In the absence of completely adequate criteria and experiments, however, one cannot be certain that denaturation produces exactly the same changes regardless of what method of denaturation is used.
- (5) The Temperature of Coagulation. The quantitative study of heat denaturation is conveniently carried out by heating an iso-electric solution of the protein which contains a considerable amount of salt. Under these conditions the protein precipitates immediately on being denatured so that the formation of insoluble protein is a measure of denaturation. As the protein is heated nothing visible happens until a temperature is reached at which denaturation and precipitation take place rapidly. This temperature varies with different proteins. It can be lowered by the addition of acid or alkali. It can be raised by the addition of salt. Edestin is not coagulated in a saturated solution of sodium chloride even if the solution is boiled.
- (6) The Temperature Coefficient of Denaturation (1, 2, 6, 7, 17, 18, 19, 20). Early workers supposed the temperature of denaturation to be very sharp and like the critical temperature which is characteristic of a change of state such as melting. One might, in-

deed, suppose that the large protein molecule, on being denatured, undergoes a change analogous to melting. Closer study of the temperatures of coagulation shows that they are not as sharply defined as melting points, at least not as sharp as the melting points of many pure substances. At any temperature, denaturation proceeds at a measurable rate which can be expressed by the equation for a monomolecular reaction. The increase in rate of reaction, when the temperature is raised 10°, instead of being two to three, as is ordinarily the case, is some six hundred. There appears to be a critical temperature of coagulation because the rate of denaturation is increased so much by a small rise in temperature. The temperature coefficient of denaturation, which is about 600 at the isoelectric point of the protein, is less in acid solution, but it is still higher than that of ordinary chemical reactions.

No other biochemical reaction is known to have a temperature coefficient of the order of magnitude of the temperature coefficient of denaturation. From the fact, therefore, that the destruction by heat of bacteria, enzymes, antibodies, and other biologically active materials, and the fertilization of the egg by heat have temperature coefficients of about 600, one is inclined to conclude that the pace-setting reaction in each case is heat denaturation of protein. This reasoning has been proven to be correct in the case of several enzymes and of the tobacco mosaic virus (20-a), which have been shown to be proteins. When these proteins are denatured, they lose their activities. It is true, nevertheless, that there may be unknown reactions which, like denaturation, have a high temperature coefficient.

2. DIFFERENCES IN THE PROPERTIES OF NATIVE AND DENATURED PROTEINS

Grossly, as we have seen, denaturation is a change in a protein which makes it insoluble, a change which can be brought about by a great variety of agents and which, when brought about by heat, has an extraordinarily high temperature coefficient. Obviously this vague description of denaturation is inadequate, especially since there are many ways of changing the solubility of a protein. A more precise, if still inadequate, description has been made possible by investigations which have been carried out only recently. For a long time the study of denaturation was neglected. Denaturation was taken to be some vague general decomposition of protein, never quite the same thing in any two cases. Furthermore,

denaturation was supposed to be irreversible, and hence of no biological interest. We shall now see that denaturation can be characterized quantitatively by definite changes in chemical and physical properties, and that under favorable conditions denaturation is readily and completely reversible.

- (1) The Nitroprusside Test (21, 22, 23, 24, 25, 26). The nitroprusside reagent gives a pink color with simple —SH compounds such as cysteine and with coagulated proteins which contain cysteine. Coagulated proteins which contain cysteine but not cysteine do not give the nitroprusside test. They give the test when a cyanide solution is added. The cyanide solution changes the —S—S— to an —SH group. Native proteins either do not give the nitroprusside test at all or give a test which is fainter than that which they give when coagulated. Qualitatively, then, denaturation results in the appearance of reactive —SH and —S—S— groups.
- (2) Estimation of -SH Groups (28). In the direct method for estimating protein -SH groups, the groups are oxidized by the addition of cystine to the solution and the cysteine which is formed is estimated by a sensitive colorimetric method. Under the conditions chosen only -SH groups are oxidized by cystine. In the indirect method the reactive -SH groups are oxidized by cystine or ferricyanide ions or inactivated by iodoacetate ions. The reagent is removed and the protein is hydrolyzed. The extent to which the cysteine content of the hydrolysate of the treated protein is less than the cysteine content of untreated protein is a measure of the number of -SH groups abolished by the treatment.

When cysteine is oxidized to cystine, two molecules of cysteine join to form one molecule of cystine. Exactly what happens when the -SH group in proteins is oxidized is not clear. It is not known whether an -SH group of one molecule combines with an -SH group of the same molecule or of another molecule.

- (3) Estimation of -S-S- groups (28). The number of -SH groups present in the protein is estimated. The -S-S- groups are reduced by thioglycollic acid. The excess of the acid is then removed. Finally the -SH groups are again estimated. The increase in the number of -SH groups due to the reduction treatment is a measure of the number of -S-S- groups which have been reduced.
- (4) Results (28, 29). The hydrolysates of some proteins contain only cysteine, of others only cystine, of still others both cystine and cysteine. In the cases so far studied the denatured protein has a number of reactive -SH groups which is equal to the number of

cysteine molecules in the hydrolysate. The number of reactive -S-S- groups is equal to the number of cystine molecules in the hydrolysate. That is, all of the -SH and -S-S- groups of proteins are accessible and reactive in denatured but unhydrolyzed proteins. This has been shown to be true of proteins denatured by heat, trichloroacetic acid, urea, ultra-violet light, and shaking in air.

Native proteins have either no reactive -SH and -S-S- groups or only a fraction of those which are detectable in denatured proteins. The number of detectable -SH groups varies not only with the protein but with the pH, for the activity of the -SH groups of all -SH-containing compounds is increased by making the solution more alkaline. For instance, native egg albumin and native hemoglobin at pH 6.8 have no detectable -SH groups; at pH 9.5 native egg albumin still has no detectable -SH groups, but native hemoglobin has about half the number which is present in denatured hemoglobin. The native proteins of the crystalline lens have some detectable -SH groups even at pH 6.8. Different proteins and the native and denatured forms of any one protein are different -SHcontaining compounds, just as cysteine and glutathione are different -SH-containing compounds. The difference between the native and denatured forms is most striking when the estimations are carried out at the most acid pH at which all of the -SH groups of the denatured form are reactive, just as the difference in the ionization of two amino acids is most striking at the pH at which one of the acids is just ionized. At an extreme pH both acids are either entirely ionized or entirely unionized. In sufficiently acid solution the -SH groups of neither native nor denatured protein are readily oxidized.

- (5) -SH Groups of a Partially Denatured Protein (30). When egg albumin is half-coagulated all of the -SH groups of the insoluble fraction are reactive, none of the soluble fraction; *i.e.* the insolubility test for denaturation in this case is justified by the -SH test. The fact that all or none of the -SH groups are changed in reactivity shows, furthermore, that by the -SH test a protein molecule is either completely native or completely denatured.
- (6) Other Reducing Groups (31). Denatured proteins which have no -SH groups or whose -SH groups have been oxidized by cystine reduce ferricyanide ions; *i.e.* denatured proteins contain, in addition to -SH groups which can be oxidized by both cystine and ferricyanide ions, other weaker reducing groups which can be oxidized only by the ferricyanide ion, which is a stronger oxidizing

agent than cystine. The number of ferricyanide ions which is reduced varies with different proteins. It is greater the more alkaline the solution, the greater the concentration of ferricyanide ions, and the greater the time during which the protein is in contact with ferricyanide ions. Since the oxidation of the weaker reducing groups by ferricyanide ions does not come to an end, as does the oxidation of -SH groups, estimation of the number of reducing groups which are oxidizable by ferricyanide ions is impossible under the conditions so far used.

Of the amino acids tried, tyrosine and tryptophane alone behave towards ferricyanide ions just as the proteins do, so that the groups which are oxidized by ferricyanide ions but not by cystine are probably tyrosine and tryptophane groups.

Native egg albumin does not reduce ferricyanide ions at pH 9.5. Denatured egg albumin whose –SH groups have been oxidized by cystine does reduce the ferricyanide ion at pH 9.5. In other proteins the difference between the native and denatured form is not so marked. In general, the reactivity of all of the groups which reduce ferricyanide ions, like the reactivity of the –SH groups alone, varies from protein to protein and is increased by alkali and by denaturation of the protein. Denaturation seems to cause a general activation of protein groups.

A cysteine-containing denatured protein whose –SH groups have not been oxidized reduces more of a ferricyanide solution under given conditions than the same protein with its –SH groups previously oxidized. The difference is more than can be accounted for by the –SH groups alone. Apparently the native –SH groups can catalyze the oxidation of other protein-reducing groups. This result shows that the so-called reactivity of protein groups is a function of the whole chemical environment. All of the peculiar groups of proteins may be more reactive in the living cell with its enzymes than they are ordinarily supposed to be.

(7) Titration Curves (20, 32, 33, 34, 35). Denaturation of a protein does not seem to cause any great change in the titration curve of the protein. The data of different investigators, however, are not in perfect agreement. There are three technical difficulties in comparing the titration curves of native and of denatured proteins. Denatured protein is insoluble near the isoelectric point. The acid and alkali which is used to titrate native protein may denature the protein. Alkali, in particular, may cause changes in the protein apart from denaturation.

(8) Conjugated Proteins. (a) Hemoglobin (36, 37, 38). The iron pigment, reduced heme, can combine with native globin to form native hemoglobin and with denatured globin to form denatured hemoglobin or globin hemochromogen. The compound of reduced heme and native globin in neutral solution is a "firm" compound. The compound of reduced heme and denatured globin in neutral solution is a "loose" compound. Reduced heme in native hemoglobin can combine reversibly with molecular oxygen. Reduced heme in denatured hemoglobin cannot do so. The spectra of native and denatured hemoglobin are entirely different, although the protein itself does not absorb visible light (see Chapter X). Thus the prosthetic group, reduced heme, which is combined with globin to form a conjugated protein, can serve as an indicator of the denaturation of the protein to which it is attached.

Other denatured proteins besides denatured globin can combine with reduced heme to form hemochromogen. The same proteins in their native forms cannot form hemochromogen (39).

(b) Visual Purple. The primary photochemical reaction in vision is the change of visual purple to visual yellow. The pigment of visual purple, a carotene pigment related to vitamin A, cannot be extracted with benzene, but extraction of the pigment is possible after visual purple has been altered by light. It has been concluded that in visual purple the carotene pigment is joined to a protein, for when visual purple in solution is subjected to denaturation procedures, such as heating, which presumably alters some protein present, the carotene pigment becomes extractable with benzene, just as it does when visual purple is exposed to light (40). It has been suggested further, that the photochemical change in the intact retina is denaturation by visible light (41). Visual purple has not yet been prepared in pure or even in approximately pure form, so that there is not, as yet, direct chemical evidence of its protein nature or of its denaturation by heat or light.

In general, only a few conjugated proteins have been prepared in pure form and the denaturation of conjugated proteins other than hemoglobin has not been studied in detail. This is unfortunate, for the presence of a prosthetic group, especially of a prosthetic group

¹ In acid solution there is both denaturation of the protein and loosening of its bonds with heme. Whether in this case the loosening of the bond is due to the acid or to the denaturation is not known. The indications are that in all conjugated proteins of the hemoglobin type in which the prosthetic group is firmly bound to the protein, the prosthetic group can be separated from the protein only when the protein is denatured.

with convenient optical properties, greatly facilitates the study of denaturation.

It would be interesting to know in particular whether the pigments which in nature catalyze the transfer of light energy are in general attached to proteins, whether these pigmented conjugated proteins are denatured by light, and whether the reversal of such denaturation is coupled with endothermic reactions. The suggestion that chlorophyll is attached to a protein is indeed not a new one. But this suggestion has not been taken seriously in an experimental way. The usual procedures for preparing chlorophyll involve exposure to light and to reagents which ordinarily cause denaturation.

(9) Optical Properties. The X-ray analysis (42) of fibre proteins such as the hair protein, keratin, shows them to consist of parallel polypeptide chains folded at right angles to the long axis of the chains. When the fibre is stretched the chain is extended. When the stretching force is released, the fibre contracts and the original folds are restored. Native globular proteins, in contrast, consist not of parallel chains but of polypeptide chains coiled into definite non-parallel configurations. Presumably the configurations of both the straight and coiled chain proteins are maintained by definite cross linkages. The exact nature of these linkages cannot be determined at present from the X-ray photographs. When a globular protein is denatured, the X-ray analysis shows that the protein becomes more like the keratin type, that is, there is on denaturation a general uncoiling of the polypeptide chains and a complete change from the original configuration of the native molecule. Furthermore, coagulated protein, like keratin, can be stretched elastically. Unfortunately, nothing more detailed can be concluded from the X-ray data alone, because of the present limitations of the X-ray technique and because as yet extremely little experimental work has been done on globular proteins first in the unchanged native state, and then denatured as shown by the tests already described, but not modified further. The general conclusion from direct X-ray evidence that denaturation involves uncoiling, however, is of great importance and, as will be shown later, is in harmony with all the chemical evidence.

The ultra-violet spectrum of denatured globin in acid solution is not very different from that of native globin in neutral solution (43). The absorption of ultra-violet light by egg and serum albumins is increased when the protein is heated in acid solution (44).

The optical rotation and refractivity of egg albumin is increased by heating (45, 46, 46-a). When muscle globulin is denatured, its double refraction of flow is destroyed (47).

- (10) Enzymes. When the proteolytic enzymes pepsin (48, 49), trypsin (50, 51), and carboxypeptidase (52) are completely denatured, as determined by solubility tests, they are completely inactivated. When they are half denatured, they are half inactivated. These results confirm the all or none character of denaturation and show the reliability, under certain conditions, of the change in solubility as a criterion of denaturation.
- (11) Digestibility. It is generally stated that coagulation of proteins increases their digestibility. But there have been few studies of the effect of denaturation on digestibility in which the denatured protein was in solution at exactly the same pH as the native protein, and in which both forms of protein were free of antienzyme. Furthermore, different proteins behave differently. Native hemoglobin is not digested at all by trypsin, whereas denatured hemoglobin, under the same conditions, is digested readily. The digestibility of egg albumin by trypsin is increased by denaturation of the protein (53). Edestin is digested readily by trypsin even when it is native.
- (12) Molecular Weight. As will be pointed out later, the equilibrium between native and denatured hemoglobin in neutral salicylate solution and between native and denatured trypsin in acid solution is independent of the protein concentration. This means that in these cases denaturation involves no change in molecular weight.

The osmotic pressures of various denatured proteins in concentrated urea solutions have been measured (54, 55, 56, 57). The osmotic pressures of the denatured forms of egg albumin, serum albumin, sheep and hog hemoglobin and globin are the same as the osmotic pressures of the native forms in the absence of urea. The osmotic pressures of the denatured forms of ox and horse hemoglobin and globin are twice the osmotic pressures of the native forms in the absence of urea. This increase in osmotic pressure is probably due to a splitting of the molecule by urea which is independent of denaturation. Urea in four molar concentration does not denature horse hemoglobin yet halves its molecular weight (58).

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(13) Crystallizability. Many native proteins have been crystallized. As yet no denatured protein has been crystallized. Protein

crystals can, however, be rendered insoluble by heating or other denaturation procedures without loss of crystalline form (27).

- (14) Viscosity (59, 60). If a protein solution is heated under conditions close to those under which the denatured protein is insoluble there is a gross increase in viscosity. A one per cent solution may yield a clear gel. This gross increase in viscosity is due to aggregation of protein molecules and can be imitated without denaturation. There is, however, a definite and quantitatively reproducible increase in viscosity on denaturation even when there is no aggregation. A concentrated urea solution containing hemoglobin is more viscous when the protein is denatured than when it is native. Osmotic pressure experiments show that the hemoglobin is not aggregated in urea solution.
- (15) Specificity. The native hemoglobins of different species of animals can easily be distinguished by their spectra. The denatured forms of hemoglobin cannot be distinguished by the same test (36). Similarly, there is immunological evidence that the denatured forms of proteins have a less marked species specificity than the native forms (61).
- (16) Changes in Protein Properties Caused by Ultra-violet Light (12, 13, 62, 63). Proteins can be denatured by ultra-violet light at room temperature. The denatured protein is insoluble at its isoelectric point. All of its -SH groups are reactive. If egg albumin, however, is irradiated with ultra-violet light at zero degrees, the irradiated protein is not insoluble at its isoelectric point. The reactivity of its -SH groups and its other chemical properties have not yet been measured. If the irradiated protein is heated at any time after the irradiation has been stopped, then the protein is converted into insoluble protein, and the rate of this thermal process has a high temperature coefficient. This formation of insoluble protein from protein irradiated in the cold, however, takes place at room temperature i.e., at a much lower temperature than the ordinary heat denaturation of egg albumin. Thus the denaturation of egg albumin by ultra-violet light has been separated into two distinct reactions, a photo-chemical change which takes place in the cold, and a thermal change which takes place in the protein which is modified by irradiation.

Paramecia live very well at a temperature of 32°. They are killed by ultra-violet light at 28°. They are not killed by short exposure to ultra-violet light at 18°. If, however, the protozoa which have been irradiated at 18° are brought to 28° they die within an

hour after the irradiation. If they are brought to 18° five hours after irradiation they do not die. In the living cell, but not in egg albumin, the effect of irradiation can, with time, either be reversed or abolished in some other way (64).

(17) Visible Light Plus a Photosensitizer. Proteins can be denatured by visible light plus a photosensitizer (65). It is not known whether this kind of denaturation, like denaturation by ultra-violet light, can be divided into two stages; whether oxygen is required, as it is for the lysis of red blood cells by visible light plus a photosensitizer (66); or whether any of the pigments which exist in nature attached to proteins can act as photosensitizers for the denaturation of the proteins to which they are attached. Hemoglobin, although it can absorb visible light, is not denatured by visible light (66).

It has been claimed that highly purified serum albumin is denatured by strong sunlight even in the absence of a photosensitizer, and even when ultra-violet and infra-red radiations are excluded (67).

(18) Surface Coagulation. If a solution of egg albumin is shaken with air, insoluble protein is formed which has the general properties of heat coagulated protein (14, 15). The first step in coagulation by shaking is the spreading of the protein at the air-water surface to form a monolayer. Whereas egg albumin in solution is a globular protein, the protein in the surface monolayer has been opened and its thickness is that of a single polypeptide chain (68). Until recently it was supposed that protein spread on a surface is completely denatured. Pepsin (68-a) and urease (68-b) after being spread, however, are still active. This suggests that spreading does not involve complete denaturation since enzymes become inactivated when denatured. It has not been shown, however, that the pepsin which is active after being spread is still in the form of a monolayer or that urease in a monolayer has more than a small fraction of its original activity. See p. 444.

There is good evidence that the membrane of the mackerel egg contains both lipid and protein and that some of the peculiar properties of the membrane are due to the fact that it contains protein spread at the surface (69, 70).

(19) The Denaturation of Myosin. The muscle globulin myosin consists of elongated molecules which are soluble in strong salt solution but which exist in muscle as precipitated micelles. There is considerable evidence that the contraction of muscle is due to the contraction of these micelles.

The amount of protein which can be extracted from muscle by strong salt solution is decreased when the muscle goes into rigor (70). A similar but less marked change in extractibility takes place when muscle is tetanized (71). The change in extractibility caused by tetanization is reversed when the muscle is allowed to recover. It has been shown that the change in extractibility in rigor is due to the fact that all the myosin in the muscle has become insoluble (72). On tetanization a part of the myosin becomes insoluble.

When the sea urchin egg is fertilized there is likewise a diminution in the amount of protein which can be extracted by strong salt solution, again due to the change in solubility of a particular protein fraction (72-a).

The change in solubility which has been described involves no increase in the number of reactive —SH groups. When either the original or the altered myosin or egg protein is subjected to a typical denaturation procedure then all of the —SH groups are activated (72-a, 72-b).

If water is removed from myosin or the labile egg protein by drying in vacuum or by freezing, the protein becomes insoluble in salt solution but there is no activation of -SH groups. In other words, by drying a change has been produced which is similar to that which takes place in rigor and in fertilization of the egg.

The heat denaturation of myosin suspended in isotonic potassium chloride solution can be separated into two distinct reactions (72-c). In the first reaction, which has the same high temperature coefficient as ordinary denaturation, there is only a slight increase in the number of active —SH groups but the myosin is converted into a form which is insoluble in strong salt solution. In the second reaction, which takes place at a higher temperature and has a much lower temperature coefficient, new —SH groups appear.

If muscle is heated, a reversible thermal contraction takes place at a temperature characteristic of the particular muscle used. Heating the muscle to a higher temperature results in an irreversible contraction. The temperature at which reversible thermal contraction takes place is the same as the temperature of the first reaction in the heat denaturation of myosin. The temperature at which the irreversible thermal contraction takes place is the same as the temperature of the second reaction in the heat denaturation of myosin.

The conclusion from all these experiments is that the change which myosin undergoes when it is converted into an insoluble but not completely denatured form is the change which it undergoes in muscular contraction.

The results which have been summarized dispose of the notion that denaturation is some vague, indefinite decomposition of protein. In general, a protein molecule is either native or denatured. The denatured form differs from the native form in definite, measurable ways. When the extent of denaturation can be estimated quantitatively by several different methods, the results given by different methods usually agree. The groups whose properties are changed on denaturation of the protein are many and varied. Indeed it is reasonable to conclude that the properties of all protein groups are changed on denaturation, and that the changes will be demonstrated as soon as methods for the study of these groups become available.

3. THE REVERSIBILITY OF DENATURATION

(1) Reversibility. When egg albumin is denatured by acid and the acid is then neutralized, the denaturation is not reversed by the reversal of the denaturation procedure. Instead the protein precipitates. This observation led to the conclusion that denaturation is irreversible. When the same experiment is carried out with hemoglobin the same result is obtained. If, however, before the neutralization of the acid which was added to the hemoglobin is completed, the protein is allowed to stand for a few seconds in a solution just sufficiently acid or alkaline to prevent precipitation, then on complete neutralization, some two-thirds of the protein remains in solution. The soluble protein can be crystallized and it has the solubility, the spectrum and the affinity for gases possessed by ordinary native hemoglobin. These observations led to the conclusion that the denaturation of hemoglobin can be reversed (36, 73, 74). The results are not due to the fact that hemoglobin is a conjugated protein for simple proteins such as serum albumin (75, 76) and globin (77, 78) behave in the same way.

The denaturation of hemoglobin (79) and of trypsin (50, 51) has been reversed completely. Hemoglobin is denatured by sodium salicylate in neutral solution. The denatured form differs spectroscopically from the native form. It is precipitated in a salt solution in which the native form is soluble. It is digested by trypsin which is not the case with the native form. When the sodium salicylate is removed, all of the hemoglobin again becomes native. The product is again soluble and non-digestible and has the spectrum of native

hemoglobin. Similarly, when trypsin is heated in acid solution, the protein becomes insoluble in a salt solution in which native trypsin is soluble and the trypsin loses its proteolytic activity. When the solution is cooled, the trypsin regains its solubility completely as well as its activity as an enzyme.

It is not known why, in some cases, denaturation is not reversible at all or only partially. Two possible reasons have been suggested. First, the denaturation procedure or the denaturation itself may result in secondary irreversible changes. It is known that acid can produce hydrolysis; heat, the splitting off of ammonia (8); and ultra-violet light, general decomposition (12). Secondly, the conditions for reversal are usually also the conditions for aggregation and precipitation of the denatured protein. This aggregation and precipitation may prevent reversal. Apart from secondary changes in the denatured protein, the very complexity of denaturation may hinder the reversal of the reaction.

(2) Equilibrium. In a neutral solution of a salicylate there is a mobile equilibrium between the native and denatured forms of hemoglobin (79). The greater the salicylate concentration, the more of the hemoglobin is denatured. The final percentage of denaturation, at equilibrium, depends only on the final concentration of the salicylate. It is the same whether one starts with native hemoglobin and adds salicylate, or with denatured hemoglobin and adds water. Similarly, there is a mobile equilibrium between the native and denatured forms of trypsin which is shifted toward the denatured form by the usual denaturation agents, viz., acid, alkali, heat, urea, and alcohol (51).

The equilibrium between the native and denatured forms of protein is independent of the protein concentration. This means that denaturation and its reversal are reactions of the same order.

From the effect of temperature on the equilibrium between native and denatured trypsin the heat of denaturation has been calculated to be 67,000 calories.

4. THE THEORY OF DENATURATION*

(1) In general, when a protein in solution is half denatured, as determined by one test, it is half denatured as determined by all other tests. In other words, the usual kind of denaturation in solution is an all or none reaction. This does not mean that denatura-

^{*} This section was written with the assistance of Dr. Henry Eyring of Princeton University.

tion does not involve a whole series of successive steps. It means that any intermediate substances formed are relatively unstable.

Denaturation by ultra-violet light, by shaking, and the denaturation by heat of myosin can take place in two stable stages. Denaturation by ultra-violet light is probably atypical. Ultra-violet light probably breaks bonds which are not broken in ordinary heat denaturation and converts the protein into a new protein which is more readily denatured by heat than the original protein. If denaturation by shaking takes place in two stages, which is still uncertain, then spreading is the first stage. Spreading, like denaturation itself, involves an uncoiling of the molecule and changes the protein into a form which is readily completely denatured. It is probable, therefore, that surface spreading involves the breaking of some of the same bonds which are broken in ordinary denaturation and that the surface forces stabilize this partially denatured protein which would not be stable in solution. Similarly the forces responsible for the structure of the myosin micelles seem to be able to stabilize a partially denatured form of myosin.

- (2) Denaturation is a monomolecular reaction. This again does not mean that denaturation consists of only a single step. It means that a single step is slower than the others and sets the pace for the reaction. The pace setting step is not necessarily the same under different conditions.
- (3) The denaturation equilibrium is independent of the protein concentration. This means that the denaturation does not involve any change in the number of protein molecules, a conclusion confirmed by osmotic pressure measurements. Any protein linkages broken in denaturation must therefore be ring linkages.
- (4) Temperature, under certain conditions, has an enormous effect on the rate of denaturation and on the denaturation equilibrium; that is to say, the heats of activation and denaturation are very high. It is not reasonable to conclude from these high heats that activation or denaturation involves the breaking of some extremely strong bond. The thermal breaking of so strong a bond would not proceed rapidly at ordinary temperatures. A more reasonable conclusion is that activation and denaturation are caused by the breaking of many relatively weak bonds. That a reaction involving many groups in a single large protein molecule should have a high heat of reaction is quite to be expected. Protein ionization which likewise involves many groups also has a high heat of reaction, if one calculates the heat per mole of protein and not per

mole of acid as is usually done. What is peculiar about denaturation as a complex reaction is that it is an all or none reaction. As a result, the ratio of completely native to completely denatured protein and the effect of temperature on this ratio can be measured. The ratio of completely ionized to completely unionized protein and the effect of temperature on this ratio, which theoretically ought to be very great, have not been measured.

Since the heat of reaction (ΔH) and the heat of activation (ΔH^{\ddagger}) are high, and the changes in free energy $(\Delta F$ and $\Delta F^{\ddagger})$ are small, the changes in entropy $(\Delta S$ and $\Delta S^{\ddagger})$ must be great.

$$\Delta F = \Delta H - T \Delta S$$

$$\Delta F \ddagger = \Delta H \ddagger - T \Delta S \ddagger$$
 (80)

 ΔH for trypsin in acid solution is 67,000 calories (51). ΔF calculated from the absolute value of the equilibrium constant at 45° is -190 calories. $T\Delta S$ is therefore 67,190 calories and ΔS is 211 entropy units. ΔH^{\ddagger} for hemoglobin in neutral solution is 60,000 calories (6). ΔF^{\ddagger} , calculated from the absolute rate of the reaction at 62.6°, is 25,000 calories. $T\Delta S^{\ddagger}$ is therefore 35,000 calories and ΔS^{\ddagger} is 105 entropy units.

In no case have the effects of temperature on both the rate of denaturation and the denaturation equilibrium been measured with the same protein under the same conditions. The exact relation between ΔS and ΔS ; is therefore not known.

(5) The breaking of many bonds with a resulting large increase in entropy, *i.e.*, in randomness or disorder, is reflected in the change of the X-ray picture and in the loss in crystallizability, decrease in species specificity, and general increase in reactivity of protein groups caused by denaturation. One would likewise expect any reaction which destroyed the definite specific structure of a protein enzyme to destroy its enzymic activity. But the enzymic activity might also be destroyed by a change from one specific structure to another.

When the theory that denaturation is the disorganization of the rigid configuration of native protein by the breaking of ring linkages was first proposed (81), the changes in X-ray pattern, in entropy, and in the general reactivity of protein groups caused by denaturation were not known. The new facts are not only in harmony with the theory but now provide its main basis. A summary of the present evidence, similar to that given here, has also been presented in a paper (82) which appeared after this chapter was first submitted.

- (6) From the effect of temperature on the rate of denaturation one can calculate the total heat of the breaking of all the bonds which are broken in thermal activation. One cannot calculate the minimum energy, i.e., the minimum wave length, of the light necessary to break each bond which is broken by light. Of the bonds broken in so-called denaturation by light, some in reality are broken thermally, since complete denaturation does not take place at 0° by irradiation alone, but only after the irradiated solution has been warmed. The bonds broken by light are probably broken one at a time and these bonds may not be the same as those which are broken by thermal denaturation in the dark. It is true experimentally that the colored protein hemoglobin is not denatured by visible light and that egg albumin is denatured by visible light in the presence of the photosensitizer, eosin. These results could not have been predicted from the effect of temperature on the rate of thermal denaturation. Their explanation requires a knowledge of the detailed mechanism of denaturation by light which does not exist as yet.
- (7) If a substance, X, such as acid or a salicylate, which causes activation and denaturation and combines with many protein groups, is added to a native protein, N, there results a complex activation equilibrium:

and a complex denaturation equilibrium:

Since X-substances, in general, increase the rate of denaturation and shift the equilibrium in favor of the denatured form, both activation and denaturation must involve an increase in combination with X, and the heat of activation or denaturation must include the heat of this increase in X-combination. The total effect of X on the heat of denaturation is the difference between the heats of reaction of X with N and D.

When an X-substance such as hydrochloric acid is added to a protein, in addition to the ionization of certain groups, there must be changes in the exact properties of other groups due to the ionization of neighboring groups and to the presence of ions in the solvent. These changes may involve the breaking of some of the

bonds which are broken by heat in neutral solution. In other words, the large protein molecule is always involved in a great variety of equilibria and it is not possible to alter any one of these equilibria without to some extent altering all the others.

These general considerations, which apply to all complex equilibria, have several interesting implications (79). First, although denaturation is usually brought about by heating to about 60° in neutral solution, or by adding acid or alkali or an organic solvent, it should be possible by means of a suitable X-substance to bring about denaturation under biological conditions. Salicylates, although of course not biological denaturing agents, at least bring about denaturation at room temperature in neutral solution. Secondly, since X-combination may involve many groups, denaturation may be very sensitive to the concentration of X. No detectable denaturation of hemoglobin is caused by a 0.15 M salicylate solution under the conditions studied, yet 0.2 M salicylate solution causes 10 per cent denaturation, and 0.4 M salicylate, 90 per cent denaturation. Finally, since the effect of temperature on denaturation depends on the nature and concentration of X, the effect of temperature on denaturation may vary greatly. A change of 10° in an acid solution of trypsin changes the trypsin from being almost entirely native to being almost entirely denatured. A change of 10°, in a neutral solution of hemoglobin containing salicylate ions, has no effect on the denaturation equilibrium, i.e., under these conditions no thermal energy is required for denaturation.

(8) The general picture of denaturation, as involving the breaking of many bonds and resulting in a large increase in randomness, is independent of any assumptions about the nature and number of the bonds broken, and hence is necessarily vague. We shall now consider only two of the more detailed structural theories of denaturation, although many theories have been suggested (34, 83).

The X-ray studies indicate that native protein consists of polypeptide chains held together in a definite configuration by side linkages, and that, in denaturation, some of these side linkages are broken and the definite configuration partially destroyed.

Two theories of the nature of the side linkages have been proposed. The hydrogen bond theory (82) assumes that the side linkages are hydrogen bonds between peptide nitrogen and oxygen bonds and between free carboxyl and amino groups. The assumption of the existence of hydrogen bonds between these groups is not arbitrary, but is based on the general knowledge that hydrogen

bonds are actually formed between groups of these types. The denaturing agents, in general, form hydrogen bonds. The heat of denaturation can be accounted for by assuming a reasonable value for the number of hydrogen bonds broken on denaturation.

After hydrogen bonds are broken, some may be reformed in various combinations so that denatured protein may be a mixture of many different substances with different pairs of groups joined by hydrogen bonds. It is not necessary, however, to make any assumption about the reformation of hydrogen bonds to account for the increase of randomness which may be due to the breaking of bonds alone.

Another theory of the side linkages, based mainly on geometrical considerations, is that there is cyclization due to enolization between = C:O- and distant -NH groups (84).

Neither the X-ray studies nor the hydrogen bond or cyclization theories permit any detailed predictions about the changes in protein properties on denaturation. Any theory which assumes the breaking of many side linkages predicts the decrease in crystallizability and, in a general way, the known changes in chemical properties.

(9) Apart from the breaking of side linkages, denaturation in any actual case includes shifts in ordinary protein equilibria. This has been studied particularly in the case of the denaturation of pepsin. Pepsin is denatured and inactivated in neutral solution. Despite the fact that the denaturation is irreversible, early experiments with impure pepsin indicated that the extent, as well as the rate of denaturation, depends on the pH. Such a result is not understandable on the basis of any theory of denaturation. Recent experiments with crystalline pepsin (85), however, show that denaturation goes to completion at all pH values and that the denaturation is a monomolecular reaction whose velocity constant, over a considerable range of pH, is inversely proportional to the fifth power of the hydrogen ion concentration. From measurements of the effect of pH on the velocity constant at different temperatures, it was concluded that the denatured protein is formed solely from an activated complex which results from the reaction of five protein groups with sodium hydroxide, that these five groups are amino groups, and that, whereas the overall heat of activation is 63,500 calories, the heat of activation corrected for the heat of ionization is only 18,000 calories. Although it is not certain that these conclusions are entirely correct, there can be no doubt of the correctness of the general point of view that the activation equilibrium includes a complex ionization equilibrium, and that the heat of activation includes a heat of ionization. Furthermore, the investigation of the part taken by the ionization equilibrium in the overall denaturation or activation equilibrium has the advantage of being concerned not with hypothetical bonds but with a reaction which is known to take place and which, in some cases, can be measured independently. In the study of the denaturation of pepsin no independent measurements of the change of ionization resulting from denaturation were made.

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 - * Review.

SECTION II. SURFACE TENSION AND FILMS

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1. SPREADING OF PROTEINS

(1) **Technique.** Substances that can be induced to spread in a monomolecular layer on water are characterized by the fact that one part of their molecules is soluble, the other part insoluble. This holds good for a very large group of substances such as fatty acids, alcohols, amines, lipids, sterols, saponins, estrin, etc., and also for many proteins.

Fatty acids of 12 to 18 carbon atoms can form a monolayer when a small amount of an ether solution has been placed on the surface of acidified water contained in a tray. The ether soon evaporates and a layer of fatty acid remains. This monolayer is formed because all of the carboxyl groups are dissolved in the water and the "paraffin" chains remain outside. We were able to show (1) that the monolayer of fatty acid can also be formed in another way; viz., by blowing out a small amount (5 cu. mm.) of a soap solution at the surface of a hydrochloric acid solution having a pH of 1.0. Both monolayers occupy the same area. This can be demonstrated by some device that enables us to determine the area of the monolayer at different pressures. By extrapolation, the area correspond-

ing to zero pressure can be found (2, 3, 4). A convenient apparatus (Fig. 1) for pressure measurements of spreading substances has been described by Gorter and Seeder (4).

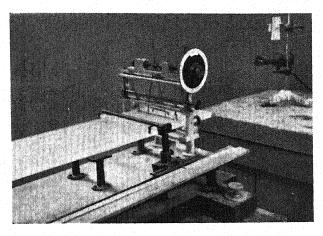


Fig. 1. Apparatus for making pressure measurements of spreading substances. (Gorter, E., and Seeder, W. A., J. Gen. Physiol., 18, 427 (1935).)

In the same way a protein can be induced to form a monolayer by blowing a small amount of a colloidal solution of the protein from a small pipette on the surface of a N/10 hydrochloric acid

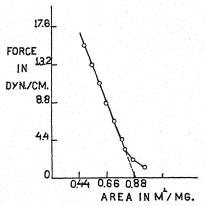


Fig. 2. Spreading of casein on HCl at pH 4.56. Temp. 15°. (Gorter, E., and Grendel, F., Proc. Kon. Akad. v. Wet., 29, 1262 (1926).)

solution. It is easy to determine the size of the area at different pressures, and to find by extrapolation the area corresponding to zero pressure (see Fig. 2). This monolayer can also be obtained on other solutions. If, however, in the case of ovalbumin, the acidity

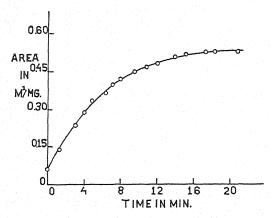


Fig. 3. Spreading of hemoglobin on HCl at pH 5.8. Influence of time on the spreading.

(Gorter, E., and Grendel, F., Proc. Kon. Akad. v. Wet., 29, 1262 (1936).)

of the hydrochloric acid solution is less than pH 3.0, the isoelectric point of this protein, considerable time is necessary in order to obtain the final spreading. In other words, if measurements are made after a few minutes, very imperfect spreading is obtained, and the

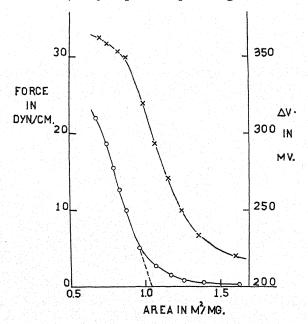


Fig. 4. Spreading of ovalbumin.

OOOO = Force-area curve, scale on left side.

XXX = Potential difference-area curve, scale on right side.

(Philippi, G. T., Thesis, Leyden (1936).)

films are not homogeneous (see Fig. 3). Under most conditions temperature favors the spreading of proteins, so that less time is necessary at a higher temperature (25°) than at a lower temperature (15°) in order to obtain monolayers on solutions of pH 3.0 (5).

Instead of determining the area-pressure curves of monolayers of protein one can also study the potential differences between the water in the tray and the ionized air above the surface, first of all when this surface is clean, and then once more when this surface is covered by a monolayer of some spreading substance such as protein (6) (see Fig. 4). For this purpose a calomel electrode is connected to the water in the tray, using an agar bridge to prevent contamination. An electrode coated with polonium is placed at a distance of some millimeters above the surface. The air between the water in the tray and the polonium electrode is made conductive by the ionization produced by the radioactive salt. The potential differences are measured with the aid of a potentiometer and an electrometer. By making this polonium electrode movable in all directions above the monolayer, one can study the homogeneity of this layer (7). This potential difference is subject to wide variations, according to the solution on which the monolayer is spread. The du Nouy method of measuring decrease of surface tension due to a monolayer of protein gives results which are identical with those given by the Langmuir apparatus, provided the monolayer of protein has been formed in the same way (8). We have been able to show that sound waves are strongly damped by a monolayer of a spreading substance. This also holds good for many proteins (9).

(2) Results with Fatty Acids. That fatty acids really spread in a layer one molecule thick can easily be shown. First of all any other position than the one assumed by Langmuir, with a carboxyl group in contact with the water and the paraffin group outside, would be extremely improbable. But the value of the size of one molecule of stearic acid, as determined by X-ray measurements, is in good agreement with that obtained by the spreading method, if the assumption is made that at a water surface the molecules of fatty acid are placed at a certain angle, just as in a crystal (10). Details of the spreading of fatty acids and the influence of temperature thereon, which have been carefully studied experimentally by Adam, are fully explained by Langmuir (2).

Whether a fatty acid can spread under normal conditions of temperature and acidity depends on the number of carbon atoms. Acids with a number of carbon atoms lower than ten do not spread; they

are too soluble. In the series of fats the same influence of the length of the chains can be shown, but, owing to the linkage of three groups to the glycerol molecule and the different polar character of the carboxylate group, the fats already spread nicely, when each chain has six carbon atoms.

(3) Results of Spreading of Proteins. From the beginning we have been struck by the thinness of protein films. We have estimated this to be 7.5 Å units (11). This value applies to a monolayer under zero pressure and under optimal condition for maximal spreading. This value has been determined on the assumption that, under these conditions, the specific volume of the protein in the film is the same as in a protein solution; viz., 0.75. If this assumption should prove to be incorrect, then the value would change to 10 Å units, which corresponds to a specific volume of 1.0. This point cannot be decided with certainty at the present time.

There is, however, one other reason for taking 10 Å units as the correct number. Philippi (6) has shown that the point in the surface-area-pressure curve and in the potential-difference-pressure curve, which corresponds to a state of maximal dehydration, has an area of 0.77 square meters per mg. If, under these conditions, a very strong pressure is applied, the correct value for the specific volume is 0.75, and once more 10 Å is found for the thickness of the protein film. A value of 10 Å units is also the distance found by Astbury between two polypeptid chains in a protein (12). In any case, the protein molecule forms a monolayer in the form of a thin plate or needle because the value, 10 Å, is much smaller than the radius of a sphere having a volume of

$$\frac{36,000}{6 \times 10^{23} \times \text{specific gravity (1.33)}} = 45,000 \times 10^{-24} \text{ cm.}^{3}$$

The radius of the sphere is 22 Å. The only possible explanation is that in a monolayer all of the peptide and the free carboxyl and amino groups are in contact with the water, and the less soluble part of the amino acids are outside of the water. This is true for all kinds of proteins at pH 1.0, and is independent of their molecular weight as given by Svedberg (13).

(4) Factors Influencing the Spreading of Proteins. The first factor which has some influence on the spreading of a protein is the technique used in forming a monolayer. Du Noüy has made use of very dilute solutions which were placed in a watch-glass and in a layer of less than one centimeter in thickness. He measured the

decrease in surface tension after having left these solutions undisturbed for a period of 8 hours. He admits that under these conditions all of the protein moves to the glass-water and water-air interface. In repeating his experiments we were not convinced that protein did not remain in the bulk of the solution. Values were found which were too small. For purposes of studying spreading, Hughes and Rideal (7) have used minute particles of dry protein, the amount of which was determined with a Nernst microbalance. We believe that this technique is subject to errors due to the fact that very small pieces of the dry material do not reach the surface. but remain included in the film. Areas are therefore found which are too small. When using our technique it is necessary to observe certain precautions in order to obtain reliable results. First of all the protein solution must be sufficiently concentrated, 5 mg./cc. being the optimal concentration. The pipette used delivers 5 cu. mm. It must be blown out by being held at a very small angle to the surface and the tip of the pipette must just touch the water surface. If dilute solutions in larger amounts are used, the spreading takes more time for completion. Smaller amounts than 5 cu. mm. cannot be measured with sufficient accuracy.

- (5) Influence of pH. This is a factor of great importance. Its influence can be studied by determining the time necessary for complete spreading on solutions of different pH, or by determining the spreading areas after the protein has been in contact with solutions of different pH for a period of one or two minutes. If determinations are made on solutions of low electrolytic strength, it is possible, with most proteins, to observe the remarkable fact that spreading is almost instantaneous at the isoelectric point, and that on both sides of this point spreading requires more and more time, so much so that almost no spreading can be obtained. In the case of very alkaline and very acid solutions, the spreading time is again short. This means that at pH values where the effective protein charge is high, the spreading tendency is at a minimum (14). The films are not homogeneous (see Fig. 5). It is interesting to note that not all proteins show this phenomenon clearly. These differences between various proteins will be described later.
- (6) Electrolytic Strength. If, instead of adding HCl to a solution having a pH of 3.0 which produces an increase in the spreading tendency, we use solutions of a salt such as potassium chloride or barium chloride, having the same strength with regard to Cl-ions as the hydrochloric acid solution, we observe the same increase of

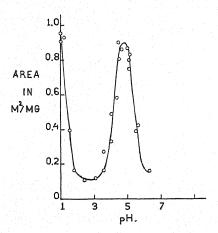


Fig. 5. Influence of pH on the spreading of ovalbumin. Readings after 1 min.

(Gorter, E., Ormondt, J. van, and Dom, F. J. P., *Proc. Kon. Akad. v. Wet.*, **35**, 838 (1932).)

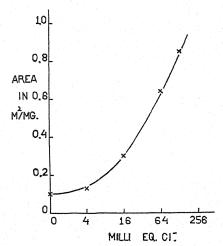


Fig. 6. Influence of Cl⁻ ions on the spreading of ovalbumin at pH 2.4.

(Gorter, E., Ormondt, J. van, and Dom, F. J. P., *Proc. Kon. Akad. v. Wet.*, **35**, 838 (1932).)

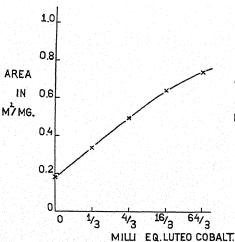


Fig. 7. Influence of luteo⁺⁺⁺ ions on the spreading of ovalbumin at pH 6.7. (Gorter, E., Ormondt, J. van, and Dom, F. J. P., Proc. Kon. Akad. v. Wet., 35, 838 (1932).)

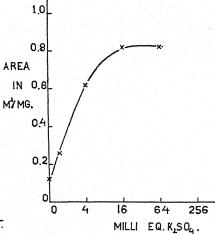


Fig. 8. Influence of SO₄⁻⁻ ions on the spreading of ovalbumin, at pH 2.4.

(Gorter, E., Ormondt, J. van, and Dom, F. J. P., Proc. Kon. Akad. v. Wet., 35, 838 (1932).)

spreading tendency (see Fig. 6). On the other side of the isoelectric point positive ions have the same influence as OH-ions.

The effect of valency can also be shown. Bivalent and trivalent ions are active in much smaller amounts than monovalent ions. Negative ions have an effect on the acid side of the isoelectric point only, whereas positive ions do not act on the acid side, but only on the alkaline side. This is shown in Figs. 7 and 8. The influence of pH and electrolytic strength of the solution can also be studied on completely spread protein films (6). The best way of

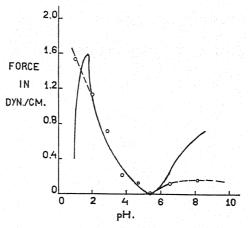


Fig. 9. Pressure exerted by a film of insulin (area 1.40 M²/mg.) (after Philippi).

○○○○ = Experimental values

Solid line = theoretical values.

(Philippi, G. T., Thesis, Leyden (1936).)

doing this is to study the pressures exerted on the balance by protein films having the same area, for instance, 1.40 square meters. This is a larger area than the area found by extrapolating a pressure-area curve to zero pressure (see Fig. 9). The pressure is minimal at the isoelectric point.

The curve is explained by the following which is taken from Philippi's publication (6). The film-pressure of completely spread protein films changes when the charge of those films is changed. It will now be shown that these film-pressure variations at constant area are due to the following factors:

- (a) the electric repulsion between equally charged film molecules or parts of these molecules;
 - (b) the electric repulsion between equally charged counter-ions;
- (c) the osmotic forces exerted by the counter-ions. These ions give an excess concentration in the neighborhood of the surface;

(d) an eventual penetration of counter-ions and other substances between the polar heads of the molecules. Such a penetration can be due either to ordinary electrostatic attraction or to a specific adsorption.

On the basis of Gouy's double-layer theory, Chapman (15) and Herzfeld (16) have calculated the drop of the interfacial tension of a water interface when it becomes electrically charged. The result of their calculation is expressed by the equation,

$$\Delta \gamma = \frac{2RT}{L} \sqrt{\frac{\overline{DRT}}{2\pi}} \cdot \sqrt{c} \left[e^{\frac{L\psi}{4RT}} - e^{-\frac{L\psi}{4RT}} \right]^2 \tag{1}$$

where $\Delta \gamma$ = the drop in surface tension brought about by the electric charge

R = the gas constant in ergs per mole

T = the absolute temperature

L = the charge of one gram equivalent in E.S.U. units

D = the dielectric constant of the solution

 ψ = the change in surface potential produced by the electric charge.

In the derivation of this formula only electric and osmotic forces have been taken into account. Any specific adsorption has been neglected. The formula can be applied to our case when this restriction is taken into consideration.

When we write

$$B = \frac{8RT}{L} \sqrt{\frac{\overline{DRT}}{2\pi}}$$

and introduce the hyperbolic sine, equation (1) becomes

$$\Delta F = B\sqrt{c} \left[\sin h \frac{L\psi}{4RT} \right]^2 \tag{2}$$

as an increase of the film pressure corresponds to a drop in surface tension of equal numerical value. Here ΔF = the difference between the film pressures of a charged and a neutral film of equal area.

In the right hand side of equation (2), ψ can be obtained from surface potential measurements, whereas all other factors are known constants.

Hence, it is possible to calculate film pressure variations from the corresponding surface potential variations without making use of any assumptions other than those involved in formula (1).

Such a calculation has been carried out using data derived from experiments with insulin. The theoretical values of the film pressure variation ΔF have been used to construct the full drawn curve of Fig. 9.

In the same figure we have indicated by points the experimentally observed ΔF values, measured at a constant area of 1.40 M^2/mg . For the calculation of ΔF the minimum pressure in the F-pH curve, situated at about pH 5.4, has been used as reference pressure. The experimental points are connected by a dotted line.

The agreement between the theoretical curve and the experimentally observed values in Fig. 9 in the pH range of 2 to 6 shows that the film pressure variations are brought about by the factors which are taken into account in equation (1), viz., electric repulsions and osmotic forces.

However, both at hydrogen ion reactions pH < 2 and pH > 6 the experimental and theoretical pressure values disagree. The deviations occur because the assumptions made in the theory do not hold true outside the pH range of 2 to 6.

If one studies the ions occupying a different place in the lyotropic series, a distinct effect is seen. The effect on the spreading of univalent ions is higher the more to the right they are placed in the series: $Cl^-Br^-I^-CNS^-$ (15). This is illustrated in Fig. 10. In this experiment ovalbumin was employed as a protein and the pH of the solution in the tray was 2.4. The influence of a cation is stronger the further to the right it is placed in the series: $NH_4^+Li^+K^+Na^+$. In this experiment pepsin was employed and the pH of the solution in the tray was 6.3.

(7) Types of Protein. The behavior of different proteins with regard to spreading can vary within very wide limits. One group is characterized by a strong tendency to spread. This is responsible for the fact that even on solutions with a very low electrolytic strength the protein spreads rapidly at the isoelectric point. Other proteins, however, do not spread rapidly at this point, but can give good monolayers either by increasing the ionic strength of the solution or by giving them sufficient time to complete the spreading. Even the same protein can behave differently, as has been shown for hemoglobin by Jonxis (18) (see Fig. 11).

In the first group a subdivision can be made, as Philippi has shown, between proteins which form a coherent film and others which spread over the whole available surface. This phenomenon can be studied with the movable polonium electrode (19). The co-

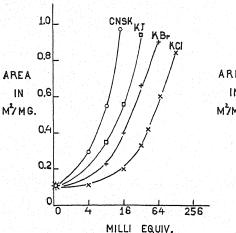


Fig. 10. Influence of different univalent anions on the spreading of ovalbumin at pH 2.4.

(Gorter, E., Proc. Kon. Akad. v. Wet., 37, 20 (1934).)

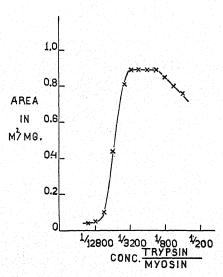


Fig. 12. Effect of varying concentration of trypsin on the spreading of myosin.

pH = 7.3. Time of action of trypsin 4 min. Temp. 38°.

(Gorter, E., and Ormondt, H. van, Biochem. J., 29, 48 (1935).)

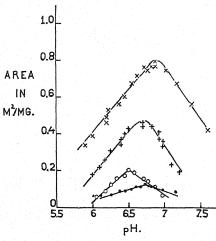


Fig. 11. Spreading of different hemoglobins on phosphate buffers (3 millimolar). Readings after 1 min. Temp. 18°.

$$\times \times \times = \text{Rabbit}$$

+++=Hog
 $\bigcirc \bigcirc \bigcirc = \text{Horse}$
• • • = Cow

(Jonxis, J. H. P., Thesis, Groningen (1935).)

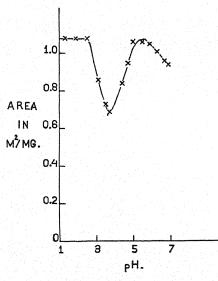


Fig. 13. Spreading of zein on buffer solutions of different pH.

(Gorter, E., and Ormondt, J. van, Proc. Kon. Akad. v. Wet., 36, 922 (1933).)

herent film has a sharp boundary towards the pure water surface.

There exist, however, other types of proteins. One group comprises those proteins which do not spread owing to a too great solubility in the solutions used. An example of this group are the protamins which show no spreading. This is apparently due to the content of diamino acids. Another representative, gelatin, which is comparable to tributyrin in the series of fats, spreads very poorly. This can be ascribed to a very large number of glycine molecules in the protein which causes it to be too soluble.

Another group of proteins consists of poor spreaders. The smaller tendency to spread is due to too great insolubility of even one part of the molecule. This can be shown by the fact that they can be transformed into good spreaders by the addition of traces of a proteolytic enzyme. This fact is illustrated in Fig. 12. Representatives of this group are myosin and fibrinogen (20). A split product obtained by the action of thrombin or trypsin on fibrinogen shows a normal behavior. The resulting substance has a pH area curve similar to that of ovalbumin (21).

Differences between various proteins other than those which consist in a greater or smaller tendency to spread at the isoelectric point can be observed. These differences consist in a smaller or larger minimum on one or on both sides of the isoelectric point. Apparently a small minimum means that the tendency of the protein in a charged condition to spread is great. This charge is higher for those proteins which have a large number of ionizable groups, and very low for those proteins which have few of these groups. In extreme cases the protein can behave as an amine having great tendency to spread on alkaline solutions only, or as an acid that spreads only on acid solutions.

Some proteins have a large number of ionizable groups; others a much smaller number. If we compare ovalbumin, which contains 36 free amino groups per molecule, with zein, which has 12 such groups, we can predict that on the acid side of the isoelectric point zein has a much greater spreading tendency than ovalbumin. The experimental results are in agreement with this prediction (22) (see Fig. 13). The same type of curve is obtained with insulin, a very good spreading substance with only a slightly diminished spreading at pH 3.0 (22) (see Fig. 14). Another example of a protein which has a very strong spreading tendency on the acid side of the isoelectric point is pepsin (23). This is in agreement with the small number of free amino groups, which have been estimated by Northrop as being only 5 (see Fig. 15).

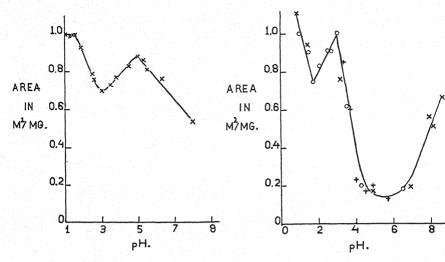


Fig. 14. Spreading of insulin on buffer solutions of different pH.

(Gorter, E., and Ormondt, J. van, ferent samples of pepsin. Proc. Kon. Akad. v. Wet., 36, 922 (1933).) (Gorter, E., J. Gen. F.

Fig. 15. Influence of pH on the spreading of pepsin. The symbols \bigcirc , \times and + indicate different samples of pepsin.

(Gorter, E., J. Gen. Physiol., 18, 421 (1935).)

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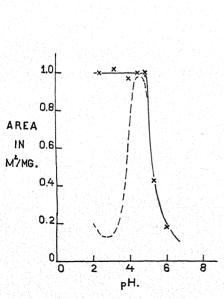


Fig. 16. Continuous curve shows spreading of ovalbumin on 1 millimolar glutathione solutions of varying pH-dotted curve shows the spreading on water at similar pH.

(Gorter, E., Ormondt, H. van, and Meijer, T. M., *Biochem. J.*, 29, 38 (1935).)

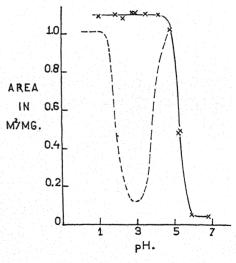


Fig. 17. The dotted curve shows the spreading of ovalbumin to which no tartrazin has been added. The continuous curve shows the area (in sq. M. per mg.) of the protein when spread on different solutions in the tray, the pH of which is indicated on the abscissa. This ovalbumin-tartrazin complex contains 5 mg. ovalbumin per mL. and is 16 mM. with respect to tartrazin, an excess of which is present.

(Gorter, E., Ormondt, H. van, and Meijer, T. M., Biochem. J., 29, 38 (1935).)

We have studied artificially prepared complex proteins of different types in order to prove the correctness of the supposition that the type of the pH-area curve depends on the ionizable groups (24). One method consists in dissolving some strongly acidic substance in water in order to study the spreading of a protein such as ovalbumin on this solution at different pH values, and to com-

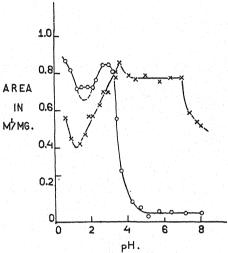


Fig. 18. The curve ○-○-○ gives the area for the spreading of pepsin (Northrop) on solutions of varying pH. The dotted line between pH 1.2 and 2.1 is the curve given by pepsin, to which no acetic acid is added as in this experiment. The curve ×-×-× gives the spreading of a pepsin-spermidin complex on solutions of varying pH.

(Gorter, E., Ormond, H. van, and Meijer, T. M., Biochem. J., 29, 38 (1935).)

pare the results with the ordinary spreading without addition of the acidic substance. Complete spreading of the protein on the acid side of the isoelectric point is obtained. Apparently all of the amino groups have combined with the acidic substance; i.e., glutathione, when the solution is one millimolar with regard to glutathione. The data are represented in Fig. 16. This same result is obtained when ovalbumin is spread on a solution containing a dye such as tartrazin. The same effect can be obtained when the tartrazin is added to the ovalbumin solution under suitable conditions so that it combines with the protein (see Fig. 17). It is obvious that on the alkaline side of the isoelectric point fixation of an alkaline substance, e.g., spermidin, gives a maximal spreading (see Fig. 18).

The spreading of a complex of ovalbumin with nucleic acid is most interesting. This acid is itself amphoteric, and one can easily understand why the complex shows the same curve as the free ovalbumin. Each amino group of the ovalbumin which combines with the phosphoric acid group of nucleic acid is replaced by another amino group of the nucleic acid which does not change its character here.

(8) Explanation of the Spreading of Proteins. In order to elucidate the spreading of proteins we have studied the spreading of

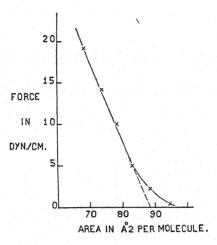


Fig. 19. Spreading of the tripeptide of α-amino-caprylic acid at pH 4.6. (Gorter, E., Meijer, T. M., and Philippi, G. T., Proc. Kon. Akad. v. Wet., 37, 355 (1934).)

different peptides. From a comparison with the spreading of fats, it was easy to predict that spreading should take place in the case of peptides which contained a great many long chain amino acids, such as leucine which has six carbon atoms. The tetrapeptide of leucine, however, was a poor spreader, comparable to tributyrin. The tetrapeptide of alanine did not spread at all. The tripeptide of α-amino caprylic acid with eight carbon atoms per chain, however, was a beautifully spreading substance. It even shows the same influence of pH and almost the same maximal spreading per milligram (1.2 sq. meters) as most proteins (25). This is shown in Figs. 19 and 20. The minima disappear by the addition of small amounts of bivalent or trivalent ions. The exact position of this tripeptide on water is well known. The peptide and the free amino and carboxyl groups are in contact with the water, the -CH₂-CH₂- chains remaining outside of the water. A model of such a peptide is shown in Fig. 21. The thickness of a layer of this tripeptide (assuming 1.0 as its specific volume in the monolayer and extrapolating to zero pressure) is 8.3 Å units. This strongly indicates that proteins do spread because their peptide groups and free amino and carboxyl groups are in contact with the water, whereas the other parts of the peptide protrude freely outside of the water. Van Ormondt has recently prepared other spreading substances that throw more light on the spreading of proteins.

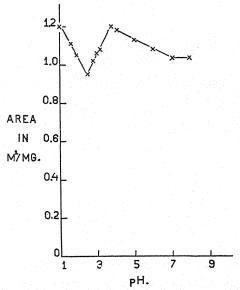


Fig. 20. Influence of pH on spreading of the tripeptide of α-aminocaprylic acid. (Gorter, E., Meijer, T. M., and Philippi, G. T., Proc. Akad. v. Wet., 37, 355 (1934).)

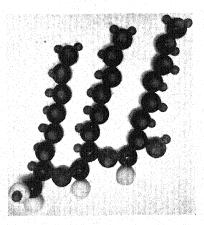


Fig. 21. Model of a molecule of a tripeptide of α-aminocaprylic acid.

(9) Does Denaturation Occur in a Monolayer of Proteins? We have been able to show (21) that pepsin or trypsin recovered from the surface of a solution on which it has been spread and redissolved in a suitable solution had lost only a very small part of its activity. This indicates that, if protein is denatured at a water-air interface, this denaturation is reversible. It seems, however, that the definition of a spreading substance itself decides this point. A spreading substance must have two parts; one soluble in water. and the other insoluble. We must therefore conclude that the protein in a monolayer is half way denatured. Complete denaturation occurs when the polar surface of the film of the protein can come into contact with another polar surface, because the outer surface of the particle will be nonpolar and the particle itself insoluble. By blowing air through a protein solution, films of a denatured type are formed because the outer surface is turned towards the air phase.

2. REFERENCES FOR CHAPTER IX, SECTION II

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SECTION III. ELASTICITY OF PROTEINS

SECTIONS III AND IV

By A. A. VAN DER DUSSEN AND L. MAASKANT (Laboratory of the Children's Hospital, Leiden, Holland)

1. HOOKE'S LAW

Protein gels are frequently elastic to a remarkable degree. By elasticity is meant the phenomenon whereby bodies which have undergone deformation due to the application of a temporary force, either partially or completely, resume their original form when the force no longer acts.

In the study of elasticity of protein gels; i.e., of microheterogeneous systems, the same ideas hold as in the case of homogeneous substances.

In the first place it was of importance to investigate the validity of Hooke's law for elastic gels and to give an answer to the question whether the moduli of elasticity calculated by applying this law give a clear insight into the elastic properties of the gel.

Hooke's law for deformations is applicable only in the case of homogeneous substances which are small compared with the length, and only as long as the elastic limit is not exceeded. The deformations brought about must be completely reversible. Hooke's law may be expressed by

$$\Delta l = \frac{Pl}{E_d \pi r^2} \tag{1}$$

where P is the deforming weight, l, the length of the cylinder, r, the radius of the cylinder, E_d , the modulus of elasticity for extension or contraction, and Δl , the shortening, when a cylindrically shaped body is deformed by means of a weight applied perpendicularly along the axis. The same law, with the same modulus, holds also for extension.

The modulus for shearing strain, E_s , is given by the equation,

$$E_s = \frac{E_d}{2(1+\mu)} \tag{2}$$

where μ is the ratio of the relative contraction of the diameter to the relative change in length. The value of μ for gelatin is 0.5. There is therefore no actual change in volume when a gelatin gel suffers extension, even when the extension is great. Since $E_s \times \text{relaxation}$ time = viscosity, the viscosity of gels must show enormous increases with increase of concentration.

As regards the method of investigation, we find extension (1) and compression (2) experiments described in the literature, and torsion measurements have also been carried out (3). The compression method is the most generally applied method and can be used for all kinds of gels.

The principle of the compression method, as described, for example, by Bungenberg de Jong and Hennemann (2), is as follows: the deformation of the gel cylinder under test is magnified by means of a lever mechanism, and the reading is made with a microscope. Thus, for example, for an experiment of short duration, the gel cylinder is loaded with a weight for half a minute and the position of a quartz fibre placed on the end of the lever between two metal supports is read off after 15 and 30 seconds, respectively. The load is then removed and the position is read off again after a similar interval.

Now various investigators have found that when the length and the diameter of an elastic gel cylinder are kept constant, the deformation is proportional to the deforming force. Thus the law of elasticity can be applied to gels provided that small deformations are used.

The elastic behavior of protein gels is not solely a function of the load as it is in the case of perfectly elastic bodies, but it is also dependent on a time factor.

In general, when a deforming force is applied to elastic gels, a deformation is obtained which increases with the time. When the deforming force is removed, the deformation disappears, but again as a function of the time. Further, a certain amount of deformation appears to be irreversible even after a very long time. The deformation of a gel under a unilateral compressive force consists of two parts: an irreversible and a reversible deformation. The latter again consists of two parts: viz., a deformation occurring immedi-

ately, and one which is a function of time. The irreversible deformation is an attendant phenomenon and does not mean that the elastic limit has been exceeded.

2. ELASTICITY OF GELS

The majority of investigations have been carried out on the elasticity of gelatin gels. Several investigators have, for example, examined the relation between the modulus of elasticity and the concentration of a gelatin gel. Leick (1) prepared gelatin gels of various concentrations and determined their modulus. He put forward the following equation which is known as Leick's law:

$$E = Kc^2 \tag{3}$$

in which c = the concentration of the gelatin, and K is a constant. Sheppard (5) found that the constants K and n, in the function $E = Kc^n$, may vary considerably for different kinds of gelatin. Thus the elasticity of gels depends considerably on the water content. For example, it is very small with casein gels which contain less than 21 per cent of the protein, and rises very quickly as the concentration rises above this figure.

3. EFFECTS OF ADDED SUBSTANCES

Leick (1) and Fraas (4) also determined the variation in the modulus on the addition of substances which affect gelation. It appears that the addition of sodium chloride reduces the modulus. Leick also found that potassium nitrate had a similar action, while potassium sulfate caused an increase in the modulus. According to Fraas, cane sugar has no effect on the modulus.

Other investigators have concerned themselves with the question as to what alterations the elastic properties undergo, starting from an isotropic mixture, when the composition of the liquid in which the gel is situated is altered. In this case the complication arises that large alterations in volume occur in a protein gel, which cannot be ignored when calculating the modulus of elasticity. Independent observations on this subject agree that in small concentrations, electrolytes have no effect on the modulus. In large concentrations, however, the modulus does alter and in salt solutions this effect follows the lyotropic series: F, SO₄>Cl>NO₃>I, CNS (6). In solutions of fluorides and sulfates the modulus increases, in other salt solutions it decreases.

As regards the effect of non-electrolytes on the elasticity of gela-

tin gels it is remarkable that the modulus of elasticity retains its original value in cane sugar solutions, while in tannin solutions, on the other hand, it falls considerably even with small concentrations. Further, it is well known that substances like alcohol (7) and glycerine (8) increase the elasticity.

Finally, it has been established for various gels that the modulus is completely reversible when it is increased, but only partially reversible when it is decreased.

There is considerable disagreement in the literature on the modulus in gelatin at various pH values. This lack of agreement is due to the various ways in which the gels changed during measurements. Gerngross (9) made gelatin gels of various hydrogen ion concentrations and determined the deformations. From his results it appeared that between pH 4.2 and 11, the modulus was constant. Scarth (10), on the other hand, started from isoelectric gelatin, which he brought to various pH values in both acidic and basic media and found that they had different moduli. The modulus was diminished especially on the acid side.

In general it can be said that the elasticity of a freshly prepared elastic gel increases as the gel becomes older. In some cases it has been noticed that the elasticity had increased to about four times the original value in a period of ten days.

As has already been stated above, in the investigation of the elastic properties of protein gels, gelatin and casein have, to a large extent, received attention from various investigators. Young's modulus has been determined under a great number of different conditions, especially for gelatin. The knowledge of these constants was in some instances indispensable for forming an idea of the structure of the gel in question.

4. THEORY OF GELS

Special mention should be made of the investigations of Sheppard (11), Poole (12), and Bungenberg de Jong and Hennemann (13). The theories of Poole and Bungenberg de Jong agree in that they represent the internal structure of the gel which must be considered as being very irregular, and when represented schematically on a flat surface, as a network of holes. In accordance with the idea of a network of particles, the hydration and the charge tend to distend the network and so increase the volume of the gel. This causes tension in the distended network. These investigators were able to test their theory by the deformations obtained by the

application of external forces. They also showed that the changes in volume observed with the gels under examination in various colloid chemical states could be explained satisfactorily from alterations in the degree of hydration and charge on the one side, and from alterations in the modulus on the other.

Attempts have been made to stipulate the conditions which are necessary in order to obtain a certain degree of elasticity in a particular substance, and, at the same time, to formulate these conditions as rigidly as possible. Although some physical theories (12–18) meet the case, they are not generally valid.

Busse (19) has finally arrived at a more general idea of the requirements which a colloid chemical system must fulfill in order that it may have elastic properties. These are: (a) long fibrous molecules; (b) weak or uniform cohesive forces around the fibres; (c) an interlocking of the fibres; and (d) a means of storing up free energy when the fibres are distorted.

X-ray investigations (20) have shown that gelatin gels consist of micelles, built up from long polypeptide chains which are loose at the ends, but bound three dimensionally in the middle. The loose ends form the means of attachment between micelle and micelle.

In elastic gels, these crystalline nuclei behave as fixed points, while the loose ends act like mobile units, so that the gel derives solidity, elasticity, and flexibility.

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SECTION IV. COHESION OF PROTEINS

Cohesion of proteins is a subject which lends itself to exact formulation only with difficulty. One speaks of loss of cohesion when protein gels or similar systems disintegrate. The forces of cohesion depend on chemical constitution and temperature. They are the same kinds of forces as those which determine solution.

1. FACTORS WHICH INFLUENCE COHESION

Wood and Hardy (1) investigated the cohesion of gluten. They placed a small quantity of gluten on the end of a glass rod and then immersed it into a beaker containing the liquid. Dilute acid quickly diminished the cohesion so that the gluten fell from the rod and gave a turbid solution.

With higher concentration of acid the cohesion first of all diminishes and then increases again. There is no simple relationship between cohesion and the degree of acidity. Salts diminish the effect of acids and alkalies on the cohesion of gluten. As a rule, the forces of cohesion in a solid jelly of gelatin do not change with the nature of the ion in combination with the gelatin (2).

The authors suggest that the loss in cohesion of gluten is caused by the occurrence of electric double layers on the surface of the gluten particles.

The subject of cohesion is treated further in the section on swelling of proteins.

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SECTION V. VISCOSITY OF PROTEIN SOLUTIONS

SECTIONS V TO VII (INCL.)

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1. DEFINITION AND METHODS OF MEASUREMENT

Any fluid offers a resistance to the relative motion of its parts when it is made to flow. This may be considered a manifestation of an internal friction in the fluid. The viscosity is a measure of the internal friction or resistance to flow. The C.G.S. unit of viscosity is named the poise. It may be defined as the tangential force per square cm. of surface required to maintain a relative velocity of one cm. per second in two layers of liquid one cm. distance apart. The viscosity is most commonly measured by observing the rate of flow of liquid through a capillary tube viscosimeter. For accurate measurement, a steady flow under a constant force parallel to the axis of the tube must be maintained. When these conditions are fulfilled the viscosity may be calculated according to the following equation:

 $\eta = \frac{\pi P r^4}{8VI} \tag{1}$

in which η is the viscosity, P is the pressure acting on the liquid, r is the radius of the capillary tube, V is the volume of liquid flowing across the whole cross section of the capillary in a unit of time, and l is the length of the tube. Two other types of viscosimeters have found considerable use, especially for the measurement of highly viscous liquids. One of these is the falling sphere viscosimeter, in which the viscosity is calculated from the rate of fall of a metal sphere through the liquid. The other is the MacMichael viscosimeter, in which the measurement is carried out by determining the twist imparted to a wire of a given diameter by the movement of the liquid around a plunger which is attached to the wire.

Usually it is rather a difficult matter to measure the absolute value of the viscosity in terms of C.G.S. units. Furthermore, the absolute values are of little importance in comparing the relationships of the viscosity of solutions. The important units for purposes of comparison are the relative viscosity and the specific viscosity. The relative viscosity is the ratio of the viscosity of the solution to that of the solvent at the same temperature. This quantity is

readily obtained simply by measuring the ratio of the time of outflow of a solution to that of its solvent with the aid of the widely used Ostwald type viscosimeter. From such a measurement the relative viscosity is given by the relation

$$\eta_r = \frac{\eta}{\eta_0} = \frac{dt}{d_0 t_0} \tag{2}$$

in which η_r is the relative viscosity, d and d_0 are, respectively, the densities of solution and solvent, and t and t_0 the times of outflow of solution and solvent. The specific viscosity is defined as the increase in viscosity produced in a given solvent by a dissolved substance. Putting it another way, it is the relative viscosity minus one. Instead of viscosity it is often useful to examine the fluidity, i.e., the reciprocal of the viscosity. The viscosity is seldom, whereas the fluidity is very often, a linear function of the concentration. According to Bingham (1) when no interaction between solute and solvent occurs in true solution or in colloidal dispersions, each successive equal increment in the concentration of the solute decreases the fluidity by a constant amount until the fluidity reaches that of the pure solute which, for proteins, is zero. It is ordinarily simpler to interpret a function which gives a linear equation than one which yields an equation of a higher mathematical order.

2. EFFECTS OF CONCENTRATION AND AGEING

A high viscosity is a property of the solutions of many, but not all proteins (2) (3) (4). This is illustrated by the difference in the relative viscosity-concentration curves of egg albumin and gelatin which are shown in Fig. 1. The viscosity of solutions of egg albumin is not vastly greater than that of the solvent, and it increases by a moderate amount in a nearly linear fashion over the concentration range shown. On the other hand, the viscosity of solutions of gelatin increases rapidly in a hyperbolic fashion with increasing concentration.

The attention of investigators has been devoted mainly to the high viscosity types of proteins such as casein and gelatin. The "true" viscosity of solutions of this group of proteins is not easily estimated. One reason for this is that the viscosity of these solutions varies with the rate of shear, or, if an Ostwald type of viscosimeter is employed, it varies with the rate of capillary flow. This phenomenon has been termed plastic flow or structural viscosity. Another reason which adds to the uncertainty of the viscosity de-

terminations among members of the high viscosity group of proteins is that certain of them show a variation in the viscosity with ageing of the solution. For these reasons it is preferable to employ the term "apparent viscosity" in connection with these proteins.

The effect of ageing on the viscosity is especially well illustrated by the behavior of gelatin. A solution of gelatin, which is cooled to

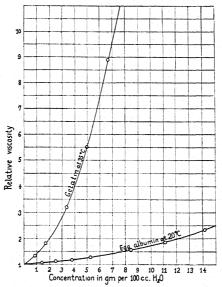


Fig. 1. Viscosity concentration curves of isoelectric gelatin at 35° and of isoelectric egg albumin at 20°.

(Kunitz, M., J. Gen. Physiol., 10, 811 (1926-27).)

below its setting temperature, shows a steady increase of viscosity with time; above the setting temperature there is a steady fall in viscosity with time. According to Sheppard and Houck (5) there does not appear to be any temperature at which the viscosity is truly independent of the time. They found that the limiting critical temperature zone, where there is a reversal from an increasing to a decreasing viscosity, is a function of the gelatin concentration, the pH of the solution, and the source of the protein. From these observations it follows that the shape of the viscosity-pH curve for gelatin is not constant, but depends largely upon the age of the solution. This is illustrated by the curves plotted in Fig. 2 which represent the influence of ageing at varying values of pH on the apparent viscosity of solutions prepared from an ash-free gelatin. The change of viscosity with age in gelatin solutions is connected with the property of gel formation possessed by this protein.

A decrease of viscosity with age has often been observed in dilute acid or alkaline protein solutions. This is perhaps due to a slow hydrolytic change in the protein. It is well known that hydrolysis by enzymes lowers the viscosity of protein solutions (6). The phenomenon of denaturation also comes to mind in connection with the time-change in the viscosity of proteins. According to Anson and Mirsky (7) and Loughlin and Lewis (8), denaturation increases

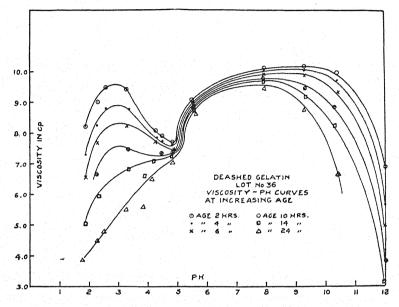


Fig. 2. The influence of ageing at a varying pH on the viscosity of gelatin solutions. Gelatin concentration, 7 per cent.

(Sheppard, S. E., and Houck, R. C., J. Phys. Chem., 34, 273 (1930).)

the viscosity of a protein. The ageing phenomenon in alkali caseinate solutions has been studied by Ettisch and Schulz (9). They reached the conclusion that the changing viscosity is related to a slow increase in the hydroxide-binding capacity of this protein. They also observed that, in the pH region of 7.4 to 11, the rate of the decrease in viscosity could be retarded by the addition of neutral salts. The drop in viscosity with time was found to be reversible even at a fairly high alkalinity.

3. EFFECT OF TEMPERATURE

Temperature has an important influence on the viscosity of protein solutions. In the high viscosity group, the apparent viscosity decreases very rapidly as the temperature is increased. This is il-

lustrated by the curve which is shown in Fig. 3. The curve represents the effect of changing temperature on the relative viscosity of a sodium caseinate solution. The rapid drop in the viscosity with increasing temperature is attributed to a decrease in the degree of the structural viscosity. According to Bogue (10), on warming gelatin to a temperature above which it sets, a point is soon reached at which the structural viscosity disappears completely. Kruyt (11)

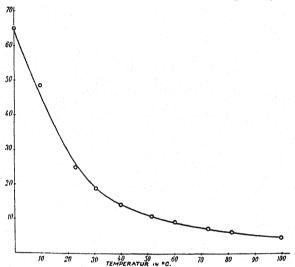


Fig. 3. The effect of temperature on the viscosity of a 9.4 per cent sodium caseinate solution. The ordinate represents the relative viscosity of the solution with respect to water of the same temperature.

(Chick, H., and Martin, C. J., Kolloid Z., 11, 102 (1912).)

states that, at sufficiently elevated temperatures, the viscosity of all proteins becomes independent of the rate of shear.

4. THEORIES OF VISCOSITY

The viscosity is a delicate means of following slight changes in protein and other colloidal solutions. However, its theoretical interpretation is still in a backward state. At present the significance of viscosity measurements is very largely a matter of speculation, or, at best, is subject only to empirical interpretation.

The viscosity equation of Einstein (12), showing the relation between the viscosity and the concentration, is the only one which is based on sound hydrodynamical principles. It is derived for a system of uncharged, suspended particles which are assumed to be rigid spheres and have a diameter which is small in comparison with the distance between the spheres. The diameter of the par-

ticles is assumed to be large in comparison with the size of the molecules of the liquid in which they are suspended. The limiting form of the Einstein equation is

$$\eta/\eta_0 = 1 + 2.5\phi$$
 (3)

in which η/η_0 is the relative viscosity of the solution, and ϕ is the ratio of the aggregate volume of the solid particles (solute) to the total volume of the solution. Equation (3) is the limiting value for low concentrations of the more complex equation,

$$\eta/\eta_0 = \frac{1 + 0.5\phi}{(1 - \phi)^2} \tag{4}$$

It is obvious that, if a solution is to conform with equation (3), the viscosity should increase in a linear fashion and only to a moderate extent with increasing concentration. Another deduction from the Einstein equation is that the viscosity should be independent of the degree of dispersion and depend only on the volume of the dissolved phase, i.e., in proportion to the concentration. This equation fails completely to account for the viscosity of protein solutions. This is not difficult to understand when one considers the wide discrepancy between the nature of protein solutions and the conditions for which equation (3) was derived. The theoretical basis of Einstein's derivation has been examined by von Smoluchowski (13). He points out that if the particles are not spherical, but instead are elliptical, polyhedral, needle-shaped, or plate-like, the constant of equation (3) will be considerably greater than 2.5.

A number of empirical equations have been developed to account for the relation between viscosity and concentration. Many of these fit the data of the viscous proteins quite closely. A basic assumption which is common to all of these equations is that the great increase in viscosity is due to a disproportional increase in the fractional volume of the solution which is occupied by the solute. The loss of effective solvent volume is attributed either to a chemical hydration of the solute, adsorption of the solvent, or a mechanical immobilization of part of the solvent through the action of osmotic forces when the solute particles form aggregates or micelles.

It is not feasible to review all of the various viscosity equations which have been proposed. Worthy of mention are the empirical equations of Arrhenius (14) and of Kunitz (15). Both of these assume that proteins in solution are hydrated to a marked degree. The Arrhenius equation for high molecular weight substances is logarithmic in form. It is

$$\log \eta/\eta_0 = \theta \frac{100W}{100 - (h+1)W} \tag{5}$$

In this equation, θ is a constant, W represents the weight of solute per 100 grams of solution, and h denotes the grams of solvent associated with each gram of solute. The term, 100W/[100-(h+1)W], which contains a correction for hydration, is assumed to be a measure of the effective concentration. Equation (5) fits the data for protein solutions remarkably well, both θ and h remaining constant over a wide range of concentration. However, the equation fails with other types of viscous colloids such as dispersions of rubber and cellulose.

The equation of Kunitz, which also fits the viscosity data of protein solutions very well, is of interest because of its resemblance to the more complex form of the Einstein equation. Kunitz came upon it as the result of a typographical error in Einstein's publication. The equation of Kunitz is

$$\eta/\eta_0 = \frac{1 + 0.5\phi}{(1 - \phi)^4} \tag{6}$$

The meaning of the symbols has already been given in connection with equation (3). Equation (6) has been used mainly to calculate values for ϕ from the viscosity data. From these values, "the degree of hydration" of the protein may be calculated. The concept of hydration held by Kunitz is not that of a chemical union between solvent and solute, but partakes more of the conception of an osmotic immobilization of the solvent. This may be gleaned from the following quotation by Kunitz (15): "The hydration of gelatin is a pure osmotic pressure phenomenon brought about by the presence in gelatin of a number of insoluble micellae containing a definite amount of a soluble ingredient of gelatin."

The explanation of a high viscosity based on immobilization of part of the solvent through the formation of aggregates or micelles has received considerable attention. Von Smoluchowski (13) states that the formation of aggregates, needle-shaped amicrons, or a foam structure will cause an apparent increase in the viscosity. In the estimation of McBain, Harvey and Smith (16), a high apparent viscosity is almost entirely due to the presence of loose ramifying aggregates of colloidal particles which immobilize within them a portion of the solvent liquid. In this connection, Staudinger (17) has stressed the rôle played by substances with long chain molecules (fiber molecules). In very dilute solutions in which the long chain molecules have sufficient freedom of movement, the long

chain molecules are believed to be in the same normal state of solution as that of any substance of low molecular weight in a dilute solution. This type is termed a sol solution. As the concentration is increased, because of the large effective volume of the long chained molecules, the total effective volume of the dissolved molecules may soon exceed the available solvent volume and the freedom of their movement becomes greatly restricted. Staudinger calls such a solution a gel solution. It has been possible to apply the ideas concerning the effect of an immobilization of the solvent on viscosity in a qualitative fashion only.

5. EFFECT OF ELECTROLYTES

The relation of electrolytes, particularly of acids and bases, to the viscosity of protein solutions, from the standpoint of the Donnan theory of membrane equilibrium, has been discussed in Chapter XIII, and will not be considered further here.

It has been observed that the effects of neutral salts on the viscosity of protein solutions arrange themselves in the order of a Hofmeister series. According to Pauli and Falek (18), at equivalent concentrations, salts with a common cation depress the viscosity to an increasing degree according to the anion series,

$$CH_3COO^- < F^- < Cl^- < NO_3^- < SCN^- < CCl_3COO^- < SO_4^-$$

The general effect of neutral salts is to produce a lowering of the viscosity of protein solutions, particularly if the protein is in an ionized state. Certain interesting exceptions to this rule have been observed with salts of polyvalent ions by Kruyt and his associates (19, 20). Kruyt and Tendeloo (19) observed that the addition of K₃Fe(CN)₆, up to a one millimolar concentration, to an electropositive gelatin solution of pH 4.4, increasingly lowered the viscosity. Further additions of this salt caused the viscosity to pass through a minimum and again increase in magnitude. With an electro-negative gelatin solution of pH 5.0, Kruyt and Galema (20) observed that salts of polyvalent cations such as magnesium, barium, and cobalt caused the viscosity to pass through a minimum, while K₃Fe(CN)₆ produced only an increased viscosity. The explanation offered for this behavior is that the polyvalent ions, by interaction with the proteins, alter the charge of the protein probably by complex ion formation. Ferrocyanide ions at first reduce the charge of the electro-positive gelatin to zero and then impart a negative charge of increasing magnitude to the gelatin as the K₃Fe(CN)₆ concentration is increased. The polyvalent cations

change the charge of the gelatin from electro-negative to electropositive. The effect of polyvalent ions in reversing the initial electrical charge on a protein has been demonstrated by means of electrophoretic measurements by Loeb (21).

The influence of an electric charge and of electrolytes on viscosity has been considered by von Smoluchowski (13) from the view point of the Helmholtz theory of an electrical double layer. He pointed out that an electric charge on moving particles produces an electro-viscous effect. From only an incomplete consideration of the problem, there was obtained the following equation relating the electro-viscosity effect to the electrical charge:

$$\eta_s = \eta/\eta_0 - 1 = \frac{5}{2} \phi \left[1 + \frac{1}{r^2 \eta_0 \lambda} \left(\frac{D\zeta}{2\pi} \right)^2 \right]$$
(7)

In equation (7), η_s is the specific viscosity, η_0 is the viscosity of the solvent in C.G.S. units, ϕ is the fractional volume occupied by the solute, r is the radius of the particle, λ is the specific conductance of the solution, ζ is the surface potential, and D is the dielectric constant. Equation (7) is intended to offer only a qualitative picture of the influence of electrolytes on the viscosity. It will readily be seen that the electro-viscous effect will disappear at the isoelectric point when ζ is zero, and will increase in magnitude with the size of the charge on the solute particles. Aside from the surface potential, the magnitude of the electro-viscous effect is largely determined by the value of the specific conductance, λ , of the solution.

On addition of neutral salts, which cause an increase in the specific conductivity, the electro-viscous effect should decrease rapidly. It has been pointed out by von Smoluchowski that only in solutions which have a low specific conductance of the order of 10^{-5} or 10^{-6} reciprocal ohms is the electro-viscous effect a factor of major importance. It would appear then that quantitatively, at any rate, this theory cannot explain the depression of the viscosity produced in most protein solutions by neutral salts.

6. RELATION BETWEEN VISCOSITY AND MOLECULAR SIZE AND STRUCTURE

Qualitative observations of a relationship between molecular size and the viscosity are numerous. As an example of this, the fact may be cited that the split products of a protein can be arranged in a series of increasing viscosity with increasing molecular size up to that of the native protein. The most important general development in this field has been made by Staudinger and his co-workers

(17). The deductions were arrived at from a study of synthetically prepared homologous long-chain organic compounds. The following is a brief account of Staudinger's views.

In general, natural substances of high molecular weight possess high viscosities. Many of these substances consist of very long molecules in which a hundred or more single molecules are bound by means of primary valencies into long chains. These are termed fibre molecules. Substances of this kind, such as the polyoxymethylenes, polystyrenes, rubber, and cellulose, are molecularly dispersed when present in sufficiently dilute solutions. Consequently, the properties of the solutions of these substances cannot be due to a micellar structure or a special solvation phenomenon, but, instead, the colloidal nature of their solutions is connected with the size and special shape of the molecules.

In dilute solutions of substances of low molecular weight with approximately spherical molecules, the specific viscosity of solutions of equal concentration is approximately constant, and is thus independent of the molecular weight. On the other hand, in the case of substances which possess long-chain molecules, the specific viscosity of dilute equimolar solutions is proportional to the molecular weight of the substance, or what amounts to the same thing, the viscosity of the solution increases with the length of the chain.

The viscosity relationships of the long-chain molecules may be represented by the equation,

$$\eta_s/C = \frac{\eta/\eta_0 - 1}{C} = K_m M \tag{8}$$

where η_s is the specific viscosity, K_m is a constant, M is the molecular weight, and C is the concentration of a primary molar solution. By a primary molecular group of a long-chain molecule is meant that structural unit which, when regularly linked end to end, builds up the long-chain molecule. For example, CH_2 is the primary molecular group of the normal paraffins and paraffin derivatives, and a primary molar solution of such a compound has, therefore, a concentration of 1.4 per cent.

Since the molecular weight is proportional to the length of the chain, equation (8) can also be written as

$$\eta_s/C = K_L L \tag{9}$$

where L is the length of the chain in Ångström units, and K_L is the corresponding constant.

Such simple relationships as are given by equations (8) and (9) hold only for homopolar compounds dissolved in non-polar solvents

when the solution is sufficiently dilute so that there are no restrictions upon the freedom of movement of the molecules.

In general, the conditions which prevail among solutions of heteropolar substances, e.g., organic electrolytes, and even of homopolar substances in polar solutions, are too complicated to fit the simple relations which are represented by equation (8). Under the latter conditions polyvalent long-chain ions may be formed from the process of one ion attaching other ions to itself. The formation of such aggregates results in a considerable and, at present, unpredictable increase in the viscosity. Staudinger's equations have been criticized by a number of workers. According to Kraemer and van Natta (22), they describe the facts only approximately under the simple conditions laid down for their use. To fit the viscosity relationship exactly, an equation of a higher mathematical order is required than the simple linear one given by equation (8).

It is readily seen that Staudinger's deductions do not have much application to proteins, since they are heteropolar substances which ordinarily are soluble only in polar solvents. However, it is of considerable interest that Polson (23) has recently claimed that he has found a constant relationship between the molecular weight of certain proteins and their viscosity increment at infinite dilution.

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SECTION VI. THE PROPERTIES, STRUCTURE, AND SWELLING (IMBIBITION) OF PROTEIN GELS

1. PROPERTIES OF PROTEIN GELS

A gel is a transparent or translucent substance which, although composed to a large extent of a liquid, retains the properties of a solid, i.e., it maintains its shape and resists deformation. It is almost invariably formed from two components, one of which is a liquid and the other a solid. The solid, which is the gelling substance proper, is called the gelator. Another important property is that substances which are soluble in the liquid component can diffuse through the gel nearly as rapidly as they would through the simple solvent. From this it follows that, except for the influence of mixing, chemical reactions can occur almost as readily in a gel as when the components are present in a fluid state. A gel that contains a high content of liquid and appears homogeneous and transparent is often referred to as a jelly. Some authors reserve the term jelly for a gel that is heat-reversible. Gels with a very low liquid content are named xerogels.

Gels are generally classified as being elastic or rigid and as being reversible or not reversible with respect to temperature. Apparently all protein gels are elastic. A good example of a rigid gel is one which is formed from silicic acid. Among proteins, gelatin is the classical example of a heat reversible gel. The heat reversibility of the gelation process persists indefinitely as long as the gelatin is not heated to above the temperature of about 70°. Gelatin maintained at a temperature over 70° for any length of time loses its ability to gel. Considerable evidence is available, e.g., a change in optical rotation, which indicates that the loss of gelling power is due to an intramolecular change of some sort in the gelatin. Fibrin and coagulated egg white are examples of elastic gels which are not reversible with respect to temperature.

The setting temperature of a heat-reversible gel is not an exact constant and does not have the same significance as the melting point of a compound. Some degree of hysteresis always occurs between the setting and melting temperatures of a heat-reversible gel. With gelatin, the setting point may be 5° to 10° lower than the melting temperature. Bradford (1) attributes the property of heat reversibility in a gel to the possession of a high temperature coefficient of solubility by the gelator.

When a gel, e.g., gelatin, is subjected to deformation, an optical

anisotropy or double refraction develops. Sheppard and McNally (2) believe that the birefringence is caused by an orientation of asymmetrical molecules or micelles in the gel during the deformation. Leick (3) found that for a given strain the double refraction increases approximately in proportion to the concentration of gelatin in the gel. He also found that the degree of birefringence is approximately proportional to the strain. Hatschek (4) observed that the optical anisotropy caused by a strain does not disappear for some time even after the external stress is removed. According to Sheppard and McNally, isotropic gelatin films are very difficult to prepare. They could make isotropic films only by drying gelatin on a mercury surface so that it is free to contract in all directions. The subject of optical anisotropy of proteins is discussed in greater detail in Section XII of this chapter.

Apparently all protein gels are elastic. Measurements of the modulus of elasticity on gelatin by Leick (3) gave 2.4 gm. per square mm. for a 10 per cent, and 29.4 gm. per square mm. for a 45 per cent gel. Poisson's ratio—the ratio of the relative contraction of the diameter to the relative change in length—was found to be about 0.5. This implies that there is no actual change in volume when a gel undergoes extension. The elasticity of gelatin gels is perfect only for small loads when these are applied for a short time. When overloaded, the gels show a fatigue.

2. THEORIES OF GEL STRUCTURE

The many theories that have been proposed to account for the structure of gels fall into the following groups: (a) gels possess a two phase liquid-liquid structure; (b) they are homogeneous single phase solid or semi-solid solutions; (c) they are two phase solid-liquid systems.

The hypothesis of Ostwald (5) that gels are emulsions of tightly packed distorted globules in a liquid medium has been pretty well discredited by Hatschek (6) who has pointed out that no known emulsion actually possesses the properties of a gel.

The conflict between the views as to whether a gel is a single phase solid solution or a two phase liquid-solid system is not so irreconcilable as might be presumed. Whether a gel is to be regarded as being composed of a single phase or of two phases depends to a considerable degree upon the properties of the gel which are subjected to examination.

It is generally agreed that gels possess a cohering, ramifying

framework which retains the liquid component and confers rigidity and elasticity upon the system as a whole. There is considerable uncertainty, however, concerning the nature and the dimensions of the fine structure of a gel. The microscopical studies carried out by the older workers, such as Hardy (7), on gels treated with fixatives appeared to demonstrate the presence of anastomosing structures of microscopic dimensions. These observations are now regarded as having been due to artifacts rather than to the inrinsic gel-structure itself. Studies with the ultra-microscope have been conflicting. With this instrument, Barrett (8) observed the formation of long intersecting fibrils during the process of blood clotting. Gelatin gels of greater than 2 per cent concentration are optically void in the ultramicroscope. In gelatin gels of lower concentration, there has been observed a grainy structure which is described as minute portions joined together in a somewhat irregular manner (9) (10).

In certain instances, interesting information on gel-structure has been obtained by means of X-ray spectrography. Katz and Gerngross (11) observed the presence of one well-defined but diffuse interference band in gels prepared from isoelectric gelatin. This offers presumptive evidence that gelatin gels contain a definite orderly arrangement of some particular grouping of the protein. Katz (12) is of the opinion that most substances of high molecular weight are composed of long chain molecules which play an important rôle in gel formation. The union of such molecules would lead to a fibrillar structure. In certain cases the X-ray spectrographs have demonstrated that solids which form gels are built up of submicroscopic crystals which are many times as long as they are thick, e.g., 500 Å long and 100 Å thick.

Proctor (13), Proctor and Wilson (14), Katz (15), Laing and McBain (16), and others, who regard gels as being homogeneous solid or semi-solid solutions of the exterior solution in the gelator, do so on the basis that the solid and liquid constituents are within the range of the molecular attraction of each other and are in chemical and electrostatic equilibrium with the external liquid, when one exists. Such a solution would constitute a single phase. Thus it has been pointed out by Proctor and Wilson that gelatin reacts as readily and as completely with acids and bases in the gel as in the sol state. Also, in the application of the Donnan theory of membrane equilibrium to account for the influence of electrolytes on the swelling of gelatin, the gelatin may be treated as a

single phase. The evidence of Katz that a gel is a single phase is based on the close parallelism between the initial swelling of proteins and the formation of a concentrated binary liquid mixture such as sulfuric acid and water.

Proponents of the view that gels are two-phase liquid-solid systems argue that two phases are present in the sense that the pores of a gel are much greater than the dimensions of a molecule. It may be pointed out in favor of the two-phase theory that, at least in a great many cases, gel formation may be considered to be the result of a partial or incomplete precipitation process. According to Bradford (1), gelation is a process closely resembling crystallization. It is usually accompanied by an evolution of heat. When crystallization from a gel proceeds to completion, there is, however, a further evolution of heat. This favors the view that gelation is a partial transformation of the gelator from the liquid to the solid state.

In the formation of a gel, a balance must be attained between stability and coagulation, if the colloidal particles are to unite to form the interlacing structure of the gel and are to be present in sufficient concentration to retain the entire liquid. On diluting a gel-forming sol, a point is reached below which the coagulated phase fails to immobilize the entire liquid, in which case a gelatinous precipitate settles out. This minimum concentration, as with gelatin or dibenzovl cystine, may be only a few tenths of a per cent. In this connection the work of Bradford (1) on gelatin is of interest. He found that ash-free gelatin is soluble only to the extent of about 0.12 per cent in water at 20°. Gelatin solutions with concencentrations of this order do not set to a gel when they are cooled, but, instead, there is a separation of the gelatin which comes down as precipitated spherites. Bradford believes that these spherites are a crystalline form of gelatin. In his estimation the process of gel formation by gelatin represents a delayed stage of crystallization, made possible by the extremely slow velocity of crystallization and the extremely high temperature coefficient of solubility of gelatin.

The micellar theory of gel structure which was proposed by Nägeli (17) is worthy of mention because of the special attention it has received in recent times, particularly by Loeb (18), Laing and McBain (16), and McBain (19). In 1858, Nägeli suggested that distensible bodies are made up of small anisotropic, crystal-like molecular aggregates (micelles) which retain their identity even when the substance is dissolved. Laing and McBain (16) came to the conclusion that the colloidal micellar units in liquid soap and

a soap gel are identical. According to these authors: "All that is necessary is to assume that the particles become stuck together or orientated into loose aggregates, which may be chance granules or, more probably, threads." This view of Laing and McBain is based on a comparison of the properties of sodium oleate sols and gels. They found that in spite of the enormous change in the viscosity

Table I

The Influence of Electrolytes on the Conductivity of Gelatin Sols and Gels

Electrolyte	Concentration N	Gelatin	Sol specific conductivity mhos×10 ⁵	Gel specific conductivity mhos×10 ⁵	Difference
		per cent			per cent
0	0	2.80	9.98	8.96	10.2
HCl	1.25×10 ⁻⁴	2.80	11.32	10.14	10.0
HCl	1.24×10^{-3}	2.75	19.10	18.65	2.3
HCI	1.14×10^{-2}	2.55	99.70	99.70	0
					446
0	0	2.70	9.44	9.73	7.6
NaOH	9.79×10^{-5}	2.70	10.00	9.90	4.1
NaOH	9.70×10^{-4}	2.65	15.55	15.00	3.8
NaOH	8.90×10^{-3}	2.45	53.25	53.25	0
0	0	2.90	9.81	8.82	10.1
NaCl	8.85×10^{-5}	2.90	12.50	12.10	3.4
NaCl	8.77×10^{-4}	2.90	22.15	21.75	1.9
NaCl	8.05×10^{-3}	2.65	98.35	97.90	0.4

(Greenberg, D. M., and Mackey, M. A., J. Gen. Physiol., 15, 161 (1931).)

involved in the transformation from sol to gel, such properties as electrical conductivity, lowering of the vapor pressure, refractive index, and sodium ion concentration remained identical, within the limits of the error of the measurements, in both the sol and gel state.

The presence of identical physical chemical properties in the sol and gel state is not true in general. With ash-free gelatin, Greenberg and Mackey (20) found that there is a distinct difference in the electrical conductivity between the sol and gel forms of the same solution. However, upon the addition of electrolytes to the gelatin, the difference in conductivity rapidly falls off to zero. The data which demonstrate this effect are shown in Table I. The experiments demonstrate that the electrochemical effects resulting from the structural changes involved in the sol-gel transformation

may be completely overshadowed by the influence of electrolytes, if they are present.

3. CHARACTERISTICS OF SWELLING

In an excellent review, Katz (12) gives the following definition of swelling: "A solid is said to swell when it takes up a liquid, whilst at the same time, (a) it does not lose its apparent (microscopic) homogeneity; (b) its dimensions are enlarged; (c) its cohesion is diminished; instead of hard and brittle, it becomes soft and flexible." Only when all three of these conditions are fulfilled may it be said that real swelling occurs. Proteins swell in water, aqueous mixtures, and liquid ammonia, but not in any of the common organic solvents such as ether, alcohol, acetone, etc.

The swelling of all substances, indeed even of a single substance, under varying conditions does not take place according to a single mechanism. Katz (12) distinguishes between what he terms intermicellar, intramicellar, and permutoid types of swelling. This division is based on a study of the changes which take place in the X-ray spectrogram during the swelling process. When the X-ray diagram remains unchanged, it may be concluded that the interior of the micelle is unaffected and that the liquid penetrates between them. This condition is termed intermicellar swelling. When the X-ray diagram changes in such a manner as to indicate that the dimensions of the elementary cell of the crystals are enlarged as a continuous function of the degree of swelling, it must be assumed that the liquid penetrates the interior of the micelle. This process is termed intramicellar swelling. When new interference lines appear on the X-ray diagram, while the old ones decrease in intensity, it must be assumed that a chemical compound is being formed by the interaction between the liquid and the solid. The process is named permutoid swelling.

The fact that the complete course of swelling of a substance may not be due to a single mechanism is brought out by Northrop and Kunitz (21). According to them, the swelling of gelatin may be readily separated into three different types: (a) swelling in acid or alkali; (b) swelling on addition of small amounts of water to dry gelatin; (c) swelling of dilute gels of isoelectric gelatin in water or in salt solutions.

Before discussing the types of swelling mentioned by Northrop and Kunitz, it is desirable to consider certain of the more striking characteristics of swelling.

Of the substances which are capable of swelling, certain of them. when put into an aqueous solution, reach a state in which no further liquid is taken up. This is a limited swelling or. in other words. such substances exhibit a swelling maximum. Most of the protein gels swell in this manner. However, a few, such as egg albumin. continue to swell without limit, finally become liquefied, and then go into solution. This process is termed unlimited swelling. There is no sharp dividing line between substances which show a swelling maximum and those that undergo unlimited swelling. The same substance under one set of conditions may show a maximum of swelling and under another set of conditions unlimited swelling. Thus Northrop and Kunitz (21) state that thin films of gelatin swell to an amount which thereafter increases only slowly with time, while large blocks do not give any indication of a maximum value but continue to swell until they are dissolved. For purposes of quantitative study very little can be done with a material unless it exhibits a stable maximum of swelling.

Hysteresis, the influence of the past history on the substance. and the slow attainment of equilibrium all tend to obscure and render the interpretation of swelling data difficult. The swelling properties of gelatin which are given below represent manifestations of these influences. It was observed by Northrop and Kunitz (21) that a block of gelatin which is brought to a definite concencentration by allowing water to evaporate from a dilute gel swells more than a similar block made by allowing a sol of the same concentration to set. The converse of this effect was noted by Arisz (22) who observed that of two gels of 10 per cent gelatin concentration, one prepared by allowing a 20 per cent gel to imbibe water to the desired value, and the other by the setting of a 10 per cent sol. the directly prepared gel took up more water than the one prepared by the previous imbibition of water. Northrop and Kunitz found that if a block of gelatin is placed in water until it has stopped swelling and is then allowed to remain in air for a short time at a higher temperature, there is no change in volume while it is in air; the gelatin will swell rapidly when replaced in water at the first temperature.

According to Sheppard and McNally (2), gelatin which is stretched or otherwise deformed while it is being dried subsequently swells anisotropically. The most rapid swelling in such a case occurs at right angles to the direction of the deformation.

An interesting phenomenon which has received widespread attention in connection with the swelling of gelatin is the so-called von Schroeder effect. Von Schroeder (23) observed that a greater amount of imbibition occurred when gelatin was immersed in water than when it was in contact with what was believed to be saturated water vapor. He found also that gelatin which was first equilibrated in water and then placed in saturated vapor lost some of its water. According to Wolff and Buchner (24) these results are due to small temperature differences between the different parts of the apparatus used in the above experiments. By using a very carefully controlled thermostat and small silvered vessels, Wolff and Buchner were able to transfer gels which had been equilibrated in water into a saturated vapor phase without incurring any loss in weight. However, with even the slightest temperature variation in the apparatus, there was a prompt loss of water from the swollen gel.

The results of Wolff and Buchner would appear to close the subject were it not for the observation of Lloyd (25) that gelatin gels, when allowed to swell in dilute solutions of acid or alkali, lose water on being transferred to an atmosphere of saturated vapor of the same temperature. The expulsion of water under these conditions is frequently so rapid that drops of liquid can be seen gathering at points all over the surface within three or four minutes after the gels have been introduced into the vapor. This loss in weight occurs if the solutions of acid or alkali are less than 0.005 molar. Gels equilibrated in solutions of a greater concentration gain in weight when placed in the vapor phase.

The explanation offered by Lloyd for this behavior is that in systems containing acid or alkali, a different equilibrium state may be expected in the vapor than in the liquid medium. In a solution of acid or alkali, the volume of the gel is determined by the balance between the elastic force of the gel framework which tends to contract the volume, and the osmotic force due to the Donnan membrane distribution of the ions in the system which tends to expand the volume. When a gel is removed from the aqueous solution, this balance is upset and the elastic force becomes preponderant, thus causing the gel to shrink.

4. EFFECT OF ELECTROLYTES

The influence of electrolytes, particularly of acid or alkali, on the swelling of proteins has been satisfactorily explained on the basis of the Donnan theory of membrane equilibrium. This type of swelling has been discussed in Chapter XIII and will not be considered further here. A lyotropic series (Hofmeister series) governs the influence of neutral salts on the swelling when the salt is present in a considerable concentration.

The electrostatic repulsion theory of Tolman and Stearn (26), to explain the influence of electrolytes on the swelling of protein

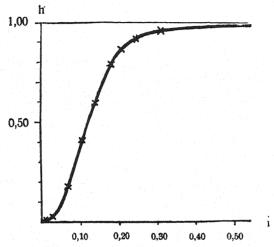


Fig. 1. The change of the relative vapor pressure with imbibition of water by asein.

Ordinate: h =the ratio of the vapor pressure of the xerogel to the vapor pressure of the pure solvent.

Abscissa: i = gms. of H_2O imbibed per gm. of protein. (Katz, J. R., Kolloid Chem. Beih., 9, 1 (1917).)

gels, still has some vogue. This hypothesis postulates that, because of the electrical charge of the same sign upon the protein particles, an electrostatic repulsion is induced which causes the gel to increase in size, the magnitude of the swelling being determined by the potential of the charge and the degree of cohesion of the protein. Obviously this theory, as well as the theory based on the Donnan membrane equilibrium, fails to account for the swelling of isoelectric proteins.

5. THERMODYNAMIC PROPERTIES OF INITIAL SWELLING

The most extensive study of the initial swelling of proteins upon the addition of liquid to a dry protein has been carried out by Katz (12). For a quantitative study of swelling it is desirable that the system under investigation be in a true state of equilibrium. This is not easy to attain because of hysteresis which is generally exhibited by swelling substances. For this reason it is usually possible to determine the equilibrium swelling curve only approximately. To minimize the resulting errors, Katz adopted the procedure of determining the curves for the uptake and the removal of water and then plotting a curve of the mean values between the two processes.

Katz measured the vapor pressures, heat changes, and volume changes of the initial process of swelling of certain proteins. The

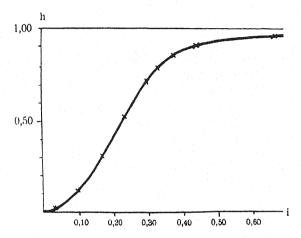


Fig. 2. The change of the relative vapor pressure with imbibition of water by crystalline edestin.

Ordinate: h =the relative vapor pressure.

Abscissa: i = gms. of H_2O imbibed per gm. of protein.

(Katz, J. R., Kolloid Chem. Beih., 9, 1 (1917).)

curve for the change of vapor pressure with swelling has a characteristic S-shape. This is shown in Fig. 1 which represents the relative vapor pressure curve of casein plotted against the degree of swelling, expressed in terms of gms. of water per gm. of dry substance. It is very interesting to note that the vapor pressure swelling curves of crystalline proteins, such as carbon monoxide hemoglobin and edestin, are the same as those for amorphous substances. This is shown by the curve for the swelling of crystalline edestin given in Fig. 2.

The swelling pressure is thermodynamically related to the vapor pressure. The equation for swelling pressure under ideal conditions is

$$P = -\frac{RT}{MV_0} \ln h \tag{1}$$

in which P is the swelling pressure, R is the gas law constant, T is the absolute temperature, M is the molecular weight, V_0 is the specific volume of the liquid, and h is the relative vapor pressure, i.e., the ratio of the vapor pressure of the gel to that of the pure solvent. When the amount of swelling is low and h is therefore small, the swelling pressure may be very large, amounting to thousands of atmospheres. As the swelling increases, however, the vapor pressure increases rapidly and the swelling pressure attains a low

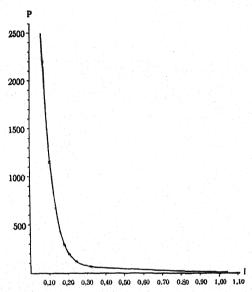


Fig. 3. Relation of the swelling pressure to the imbibition of water by casein. The swelling pressures have been calculated from the vapor pressures in accordance with equation (1).

Ordinate: P = the swelling pressure in atmospheres. Abscissa: i = gms. of H_2O imbibed per gm. of protein. (Katz, J. R., Kolloid Chem. Beih., 9, 1 (1917).)

value. The curve of the swelling pressures of casein calculated from the values of the vapor pressure is shown in Fig. 3. From a thermodynamic point of view the swelling pressure is identical with the osmotic pressure. In either case the pressure is a consequence of the fact that one component of the system is not free to permeate the total volume of the system. The swelling pressures of gelatin have been directly measured by Freundlich and Posnjack (27) and by Terzaghi (28). Using gelatin, Freundlich and Posnjack were able to measure pressures up to 6 atmospheres; Terzaghi up to 50 atmospheres. The validity of equation (1) was not directly tested in these

investigations. Because it is derived for ideal conditions, it is improbable that equation (1) represents very exactly the curve of swelling pressures.

All proteins which have been studied exhibit a strongly positive heat of swelling. The initial heat of swelling, i.e., the amount of heat developed when a very large amount of dry substance takes up one gm. of water, is about 265 small calories for casein. In the case of protein crystals which swell, e.g., edestin, the value is about 200 small calories. The heat of swelling also decreases very rapidly as swelling progresses, and is very small for protein gels of considerable water content. From a comparison of the differential heat and the change in the free energy calculated from the vapor pressure, Fricke and Lüke (29) reached the conclusion that there is an actual decrease in the entropy in the first phase of swelling. This is explained by the hypothesis that a more orderly arrangement of the water molecules is developed in the swelling substance than is the case in water.

Another interesting change which occurs during the initial swelling of a protein is a marked decrease in the total volume of the system. This becomes smaller than the sum of the original volumes of protein and liquid. The degree of volume contraction produced by a small imbibition of water is quite large; in the case of casein, it was found by Katz to reach the limiting value of 390 cubic mm. per gm. of dry solid at the point of zero water content. In a careful study of the volume contraction of gelatin, Svedberg (30) found considerably smaller decreases. For dry gelatin the volume contraction reached a maximum value of about 54 cubic mm. per gm. of gelatin dissolved in water at 35°. The contraction increased as the temperature decreased. No break in continuity was observed during the change from sol to gel. As the water content of a gel increases, the extent of volume contraction becomes quite small. The volume contraction represents a tremendous compression of the swelling components and would appear to be an expression of a very strong attraction between the protein and water.

Katz (12) arrived at the conclusion that the initial phase of swelling is in many ways analogous to the process of solution. The quantitative laws of swelling harmonize with the hypothesis that a solution is formed by the swelling components. In support of his theory, Katz points out that binary mixtures of such liquids as sulfuric and phosphoric acid, which are only slightly volatile, exhibit properties which are parallel to those observed during swell-

ing. The parallelism extends to the vapor pressure curves, the heat of mixing, and a contraction in the volume of the systems. The nature of the crystalline proteins which are capable of swelling is of considerable interest in connection with this problem. If these crystals are truly homogeneous, their swelling is presumptive evidence in favor of the solid solution theory. From the study of the X-ray patterns of protein crystals, it appears that, while the crystals exhibit interference patterns, they do not conform completely to the spectrograms of ordinary crystalline substances.

6. SWELLING OF DILUTE ISOELECTRIC GELS

The swelling properties of dilute gels of isoelectric protein (gelatin) in water or in salt solution has been studied especially by

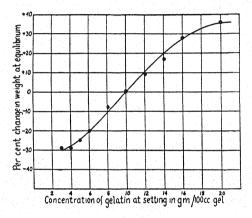


Fig. 4. Percentage change in weight of gels of various concentrations of isoelectric gelatin when placed in M/1000 acetate buffer of pH 4.7 at 5°. (Northrop, J. H., and Kunitz, M., J. Phys. Chem., 35, 162 (1931).)

Northrop and Kunitz (21, 31, 32, 33). The significant experimental findings are that, in general, the swelling increases with the temperature and with the concentration of gelatin. At 5°, gels having a greater content of gelatin than 10 per cent, when placed in water or dilute buffer solution, swell. On the other hand, those of less than 10 per cent gelatin content lose water and shrink. A gel of about 10 per cent concentration neither swells nor shrinks. This property of isoelectric gelatin gels is illustrated in the curve plotted in Fig. 4. In studying the syneresis of gels, Kunitz (33) observed that the lower the concentration of gelatin, below 10 per cent in the gel, the greater is the amount of water lost. In the complete absence of water, the process of syneresis is very slow due to the slow rate of diffusion of water through the dry surface of the solid gelatin.

However, a trace of water, when introduced on the surface either through addition or through pressure, will start a rapid diffusion of water from the gel. The same quantity of water is finally lost whether the block is immersed in a large volume of water or whether syneresis is initiated through mechanical forces such as shaking or pressures. Gels to which acid, alkali, or salt have been added, and the electrolyte subsequently removed by dialysis, exhibit a greater degree of syneresis than does isoelectric gelatin.

By means of an apparatus devised to measure directly the swelling pressure of the gelatin gels, Northrop and Kunitz were enabled

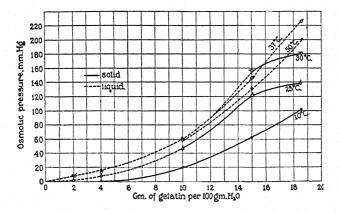


Fig. 5. Concentration and swelling or osmotic pressure of gelatin at different temperatures.

(Northrop, J. H., and Kunitz, M., J. Phys. Chem., 35, 162 (1931).)

to compare the swelling pressure with the osmotic pressure. This comparison demonstrated that the two pressures are essentially identical. This is shown in Fig. 5 by the plots of the osmotic pressure curves of gelatin sols and the swelling pressure curves of gelatin gels at various temperatures. From Fig. 5, it may be seen that when a sol sets to a gel, the swelling pressure closely follows the osmotic pressure curve of the sol.

From these studies, Northrop and Kunitz arrived at the conclusion that the swelling of dilute protein gels is mainly a process of osmosis due to a greater concentration of mobile molecules in the gel over that of the outside solution in which it is immersed. This is essentially the same theory as was adopted by Proctor and Wilson (14) and by Loeb (18) to explain the influence of acid and alkali on the swelling of proteins. Where the swelling is brought about by electrolytes, the osmosis, of course, is due chiefly to the ions of the electrolyte which are in combination with the protein in the gel

and which are not free to diffuse out. The theory of osmosis in its simple form obviously does not explain the syneresis, or negative swelling, which occurs when the gelatin concentration is less than 10 per cent.

According to Northrop and Kunitz (21), although it appears probable that very concentrated protein gels are single phase solid solutions, as postulated by Katz, it is very difficult to account for the properties of the more dilute gelatin gels on this basis. The assumption that dilute gelatin gels consist of two phases seems in much better agreement with the facts. From the results obtained by the fractionation of gelatin (21), it would appear that these dilute gels consist of at least one solid and one liquid phase and at least three components, namely, water, a "soluble," and an "insoluble" gelatin.

On the basis of the composition given above, the swelling in the gels is caused by the osmosis induced by the "soluble" component of gelatin. The amount of "soluble" gelatin in the liquid phase is large and hence the swelling increases with the temperature and with the gelatin concentration. Syneresis is caused by the elastic stress in the micelles of the gelatin. This, in turn, is determined by the osmotic pressure of the dissolved gelatin in the internal liquid phase of the micelles. As a gelatin solution cools and sets, the dissolved gelatin in the micelles becomes insoluble and precipitates. As a consequence, the force which kept the micelles stretched is diminished. This allows the elastic walls of the micelles to shrink and extrude water. The shrinking of the individual micelles brings about a contraction of the whole gelatin block.

When a gel is immersed in water, it becomes subjected to a tensile strength on swelling and to a compressive stress on shrinking, both forms of stress being proportional to the corresponding amount of swelling or shrinkage. From this it follows that the osmotic pressure of the liquid phase and the elastic stress in the micelles act against the elastic resistance of the gel. The amount of either swelling or shrinking depends upon the equilibrium between these three forces. This can be expressed by the equation,

$$P_0 - P_m = K \frac{V_e - V_0}{V_0} \tag{2}$$

in which P_0 is the osmotic pressure, P_m is the pressure due to the elastic stress in the micelles, K is the bulk modulus of elasticity, V_0 is the initial volume of the gel, and V_e is the equilibrium vol-

ume after swelling or syneresis. Equation (2) was found to hold quite well for the shrinking and swelling of a series of gels ranging in concentration from 3 to 20 per cent.

The literature on the subject of swelling is enormous. Because of lack of space it is not possible to summarize it completely. Only the salient features of the subject have been included in this presentation. An exhaustive compilation of the literature is given by Freundlich (34).

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SECTION VII. HYDRATION (BOUND WATER) OF PROTEINS IN SOLUTION

1. DEFINITION AND THEORY OF HYDRATION

The term hydration is used in chemical terminology with a variety of meanings. Very often it is used in a vague way to cover a lack of concrete ideas. This, in a large part, is due to the fact that it is extremely difficult to frame precise criteria by which the meaning of hydration, particularly as it applies to substances in solution, may be definitely fixed. It is desirable to define the sense in which the term will be used here. In this section, hydration will be employed to signify the combination of water molecules with dissolved protein in such a manner that the water becomes a part of the molecular kinetic unit of the protein. The term, "bound" water, has been widely used with a significance which is about equivalent to the one given above for hydration. From this definition it necessarily follows that water of hydration, or "bound" water, does not retain its normal solvent properties.

In its widest sense, the term hydration is used to imply that an attractive force exists at a given instant between the solute and certain of the water molecules of the solution. This may range from a feeble attraction to one sufficient to produce chemical union. There is no difficulty in determining whether or not hydration of a compound has taken place when the hydration is the result of the formation of covalent bonds. This is illustrated by the electronic equation given below for the production of ammonium hydroxide.

In this reaction the hydrate results from the formation of a hydrogen bond between nitrogen and oxygen. In the instances where the attractive forces do not lead to the formation of a chemical bond, it has not been possible so far to form exact ideas concerning the nature of hydration.

It is a difficult task to demonstrate experimentally whether a protein or, for that matter, any substance in solution is hydrated. In the crystalline state, the necessary evidence for hydration is the presence of water in the compound in a definite stoichiometrical proportion. Crystalline hydrates are known to occur among the

amino acids, examples being asparagine (1) and the dichloro- and dibromo-derivatives of *l*-tyrosine (2). However, as has been pointed out by Sidgwick (3), because a solid hydrate of a given formula separates out is no evidence that the hydrate exists in solution as a single molecule. The study of X-ray spectrograms has revealed the fact that the molecular components of a crystal-unit are packed together owing to the operation of forces altogether subordinate to those which unite the atoms in a molecule. These forces are probably due to weak electrical fields which surround every atom. Consequently, when the solid substance goes into solution, the crystal structure is destroyed and its molecular components separate again.

In solution the efforts to obtain evidence of hydration have been based largely upon the study of the deviations of the colligative properties from the laws for an ideal solution. It is now recognized that a number of factors may exert an influence on the energy state and, as a consequence, upon the character of the colligative properties of the solution. These factors include the weak attractive forces of van der Waals, dipole interaction, interionic attractions, and even electronic forces. Changes in the degree of association of the water through the mechanism of hydrogen bond formation may also be important. Even when a chemical bond is formed between a water molecule and the solute, the possibility exists that other water molecules may become involved because of their being linked to each other by means of hydrogen bonds. It is usually impossible to segregate the degree of influence of each of the above mentioned forces on the behavior of a solution. Consequently, it is understandable why the attempts to estimate the degree of hydration from the deviation of the colligative properties from the ideal solution laws have been inconclusive. A notable instance of this kind is offered by sucrose. Many efforts have been made to account for the behavior of sucrose in solutions by assigning a hydration formula to it. However, as has been pointed out by Scatchard (4), the evidence for the existence of a definite sucrose hydrate is not concordant. From vapor pressure measurements, he came to the conclusion that the hydration of sucrose apparently varied from about 7 moles of water per mole of sucrose in dilute solutions to less than 4 moles in concentrated solutions. This would appear to be an example of the difficulty of estimating the effect of each of the different forces which may influence the behavior of a solution.

In connection with the problem of hydration, it would be de-

sirable to know how the solvent properties of water are influenced by the action of each of the attractive forces enumerated above. When compound formation occurs it seems safe to assume that the hydrate water loses its solvent property. The effect of the other attractive forces on the solvent properties is difficult to estimate. In the definition of hydration and "bound" water which has been adopted in this section, it is assumed that there is a loss of solvent properties. The reason for this, as will be seen below, is because the experimental attempts to estimate the hydration of protein are based on the assumption that the "bound" water no longer functions as a part of the solvent.

The mode of combination between water molecules and a protein has been considered by Lloyd and Phillips (5) from the point of view of the electronic theory of valency. They point out that proteins in solution could become hydrated through the formation of covalent bonds between water and the oxygen, nitrogen, and hydrogen atoms of the hydroxyl, carboxyl, amino, amide, and imino groups which are present in the protein. Since the oxygen and nitrogen atoms of the protein groups are more powerful donors of electrons than the hydrogen atoms are acceptors, it is more probable that the water molecules become united to the protein molecule through hydrogen bonds. Coordination of water in this manner is characteristic of uncharged groups and should therefore be independent of the ionization of the protein.

It is a well-known fact that, in the crystal state, hydration of a salt occurs more readily if there is a difference in the electro-valence number between the cations and anions of the salt and also when there is a large difference between the sizes of the ions. Proteins are highly polyvalent and their ions are much larger than the inorganic ions with which they are combined. These factors should favor a greater degree of hydration of proteins when they are in an ionized state. An additional factor favorable to hydration of proteins when in the ionized state is that the electrical charges on the amino and carboxyl groups of the protein favor the orientation of water dipoles around them.

The mechanism of hydration postulated by Lloyd and Phillips is theoretically valid. However, as is pointed out by Pauli (6), it is purely hypothetical and it is more than doubtful that the hydrogen, nitrogen, and oxygen atoms of the various polar groups in the proteins which have been enumerated actually do coordinate with water molecules.

2. EXPERIMENTAL TESTS FOR HYDRATION OF PROTEINS

The experimental attempts to estimate the degree of hydration of proteins in solution have not yielded clear-cut results. In 1933 Greenberg and Greenberg (7) wrote: "At the present time there exists a very wide divergence of opinion regarding the state of water in biological fluids and in solutions of the lyophilic colloids." On the one hand it is held that the colloidal material in these solutions is hydrated to a high degree and that the water "bound" by the colloids does not possess its usual solvent properties (8). The opposite point of view is that all but a small fraction of the water in the biological and colloidal systems is in its usual solvent state. The trend of recent experimental evidence, as will be brought out below, greatly favors the view that proteins in solution, in terms of the definition used here, are hydrated to only a slight degree, if at all.

The confused state of the views regarding the hydration of proteins is due, in part, to the lack of precise criteria by which hydration may be defined. This is illustrated by the conclusion of Neville and Theis (9) who state: "The swelling of gelatin at the isoelectric point cannot be due to osmotic force but may be attributed to hydration—that is, to the compression of a shell of liquid about the particles which results in a contraction of the system." Since it has been satisfactorily established that the greater part of the water in a dilute gelatin gel retains its normal solvent properties, the imbibed water of the gel cannot be in the form of water of hydration in the sense that this term is used in this section. Part of the disagreement regarding the hydration of proteins is also due to differences of opinion about the interpretation of the data obtained by various physical methods which have been devised for the purpose of estimating "bound" water.

The unsatisfactory status of the subject is shown by the wide divergence in the values of the "bound" water found in the same systems by the use of different methods. The published figures for the "bound" water in gelatin solutions (see Table I) offer a good illustration of this. From Table I it is seen, and this is equally true for other systems which have been investigated, that there is a complete lack of agreement regarding the extent of the hydration of gelatin in solution.

The main experimental methods which have been used to estimate the state of the water in protein solutions will now be briefly described and evaluated.

Table I

Published Values for the "Bound" Water in Gelatin Solutions as Determined by Various Methods

Method used	Gelatin content	Gm. of "bound" water per gm. of gelatin	Refer- ence*
	per cent	gm.	
Cryoscopic with sucrose as reference substance	1 to 5	2.0 at lowest gelatin con- tent to 1.0 at highest	(10)
Freezing out method; H ₂ O measured calorimetrically		2.0	(11)
Freezing out method; H ₂ O measured with dilatometer	2 to 32	4.7 at lowest gelatin content to 0.7 at highest	(12)
Vapor pressure method		3.0	(13)
Reference substance sucrose Reference substance KCl		3.0	(13)
Reference substance NaCl		1.0	(13)
Freezing out method; analysis of			
gelatin residue	12 to 40	0.53	(14)
Contraction in volume		0.08	(15)
Osmotic pressure deviation from	1 1		
van't Hoff's law	1 to 14	4.7	(16)
Viscosity	1 to 14	7 at lowest gelatin con- tent to 3.35 at highest	(17)

^{*} Numbers refer to reference list at end of section. (Greenberg, D. M., and Greenberg, M. M., J. Gen. Physiol., 16, 559 (1933).)

The divergence of the osmotic pressure and viscosity of protein solutions from a linear relationship to the protein concentration has been explained on the assumption that the protein is extensively hydrated. Both the osmotic pressure and the viscosity follow the path of a hyperbolic curve with increasing concentration of protein. If extensive hydration of a protein occurs and the water of hydration has lost its normal solvent properties, it is readily seen that the protein concentration in terms of the total water content does not represent the true concentration of the protein with respect to the solvent, water. The true concentration must become increasingly greater as more and more water becomes "bound." This would account for the types of osmotic pressure or viscosity curves that are obtained in protein solutions. However, as has been pointed out in Chapter VIII, deviations of the osmotic pressure from a simple linear relationship are probably due to the effect of attractive and repulsive forces between the ions and molecules present in the solution upon the thermodynamic activity of the protein. Alternative explanations to hydration, e.g., the mechanical immobilization of the solvent, have been proposed to explain the viscosity curves (see Section V).

The behavior of protein solutions during freezing has been made the basis of a method for estimating "bound" water. As developed by Rubner (18), Thoenes (11), and Robinson (19), the total water content of the system is determined on an aliquot portion, the specimen is frozen to -20° , and the amount of water which has frozen out under these conditions is estimated by calorimetric measurement of the latent heat of fusion of the ice so formed. The difference between the total water and the amount frozen is taken as a measure of the "bound" water in the system. Rubner selected -20° because he considered that at this temperature all of the water which was associated with the salt (chiefly NaCl) in biological systems would be frozen out. The temperature of the eutectic mixture of ice and sodium chloride is -23° . During a dilatometric study of the freezing of gelatin, Jones and Gortner (12) observed that all of the water which would freeze was frozen at -6° , no additional water being frozen even when the gelatin was cooled to -50°. The freezing of gelatin gels has been especially carefully studied by Moran (14). He froze discs of a gelatin gel very slowly so that all of the ice formed on the surface. At the end of the procedure it was found that 0.53 gm. of water per gm. of gelatin had remained unfrozen at -20° . Kistler (20) has made a critical examination of freezing as a method for estimating "bound" water. He came to the conclusion that the discrepancies which appear in the results of the various workers who have used this method (see Table I), and the reason that all of the water of a protein system does not freeze out, are due to undercooling. He demonstrated that water in isolated droplets can readily be undercooled and remain unfrozen at the temperature to which specimens are customarily subjected in the freezing-out method of determining "bound" water. Kistler points out that, in biological tissues and protein systems, conditions are quite favorable for the isolation of small masses of water which may remain in a liquid condition indefinitely at temperatures far below the freezing point.

The effect of a reference substance on certain of the colligative properties of protein solutions, namely, lowering of the freezing point (21) and of the vapor pressure (22), has been developed into methods for determining the hydration of biological colloids. The use of a colligative property of solution for estimating water of hydration depends upon a definition of the behavior of the "free"

and "bound" water with respect to this property. Hill (22), who developed the vapor pressure method for estimating hydration, states that the term, "free" water, may be defined in various ways and that the different definitions do not necessarily coincide with each other. It is readily seen that any definition for "free" water is inherent in the physical property which is being measured. Since all of the colligative properties of a solution are thermodynamically interrelated, and, therefore, with sufficient data, are calculable one from the other, Greenberg and Greenberg (7) have proposed the definition that the weight of water in one gm. of fluid or tissue which can dissolve substances added to it with a normal change in a colligative property is the "free" water.

The manner in which a colligative property of a solution is employed for the estimation of "bound" water may be seen from a description of the cryoscopic method. In the application of this method, the freezing points of the initial protein solution and of the protein solution after a definite quantity of a reference substance has been added are measured. The lowering of the freezing point of the reference substance in pure water at a concentration equivalent to its concentration with respect to the total water of the protein system is also estimated. If dr is the freezing point lowering of the protein solution plus the reference substance, dp that of the initial protein solution, and $d_{\text{H},0}$ the freezing point depression of the reference substance in water, then if $dr - (dp + d_{H_{2}O})$ is equal to zero, all of the water is functionally free. If some of the water is "bound" the summation of the above terms has a positive value. The substances that have been mainly employed as reference substances in this type of procedure are sucrose, urea, and sodium and potassium chloride. The results of the study of hydration by the lowering of the freezing point or vapor pressure methods led Hill (22), Grollman (13), and Sunderman (23) to conclude that the water of blood, blood corpuscles, muscle, egg white, and various other protein solutions is nearly all "free." In a re-examination of the cryoscopic method for the determination of "bound" water, Gortner and Gortner (24) admit the possibility of appreciable errors in its application, particularly because of uncertainty in the correction to be applied for undercooling.

The last of the methods for the detection of hydration of proteins that need be considered also depends upon the use of a reference substance. In this method the distribution of the reference substance is determined by ultrafiltration. As pointed out by

Table II

Test for "Bound" Water in Gelatin and Casein Solutions by Means of Varying Quantities of Glucose as Reference Substance

No.	Glucose in original protein-free solution per 100 ml.	Glucose in ultra- filtrate per 100 ml.	"Bound" water per gm. of protein
	5.0 per cent ge	elatin in water+glucos	se
	mg.	mg.	gm.
1	100	101	0.20
2	200	199	-0.10
3	300	301	0.07
4	400	400	0.00
5	500	502	0.08
Average			0.05
6 per ce	nt casein in aqueous solu 5 millimol	tion containing 5 milli s NaCl per 100 ml.	mols NaHCO ₃ and
6 per ce			mols NaHCO, and
	5 millimol	s NaCl per 100 ml.	
1	5 millimol	s NaCl per 100 ml.	0.00
1 2	5 millimole 100 200	s NaCl per 100 ml. 100 200	0.00 0.00
1 2 3	5 millimole 100 200 300	s NaCl per 100 ml. 100 200 301	0.00 0.00 0.05

(Greenberg, D. M., and Cohn, W. E., J. Gen. Physiol., 18, 93 (1934).)

Greenberg and Greenberg (7), if water of hydration has lost its normal solvent properties, a crystalloidal reference substance when added to a colloidal solution should then distribute itself only in the "free" water of the solution. If a portion of the solution is ultrafiltered through a membrane which permits the passage of the solvent and other crystalloids only and holds back the colloidal constituents, the concentration of the reference substance in the ultrafiltrate liquor becomes a measure of the "free" water in the colloidal solution provided certain criteria are met. From such an ultrafiltration experiment, h, the "bound" water per gm. of protein, may be calculated according to the equation,

$$h = \frac{1}{P} \left(1 - \frac{C_T}{C_u} \right) \tag{1}$$

in which P is the amount of protein per gm. of total water, C_T is the concentration of the reference substance per gm. of total water

in the system, and C_u is the concentration of the reference substance per gm. of water in the ultrafiltrate.

To obtain valid results with this method, the reference material must not react with or be adsorbed by the protein, and the reference substance cannot be an electrolyte when the protein carries an electric charge (see Chapter XIII for reasons).

A careful study by this method indicates that only a very small fraction of the water in protein systems can be "bound" to the protein. To illustrate this, the data obtained by Greenberg and Cohn (25) on the ultrafiltration of solutions of gelatin and casein using glucose as a reference substance, are given in Table II. The data in the table indicate that water of hydration can be present in gelatin or casein solutions only to a minute extent. It is highly improbable that each gm. of protein can contain several gms. of "bound" water (cf. Table I). From the discussion which has been given above it is readily seen that hydration does not appear to be an important factor in determining the properties of protein solutions.

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CHAPTER IX (CONT.)

SECTIONS VIII TO XI (INCL.)

BY CARL L. A. SCHMIDT

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SECTION VIII. PROPERTIES OF CRYSTALS

1. CRYSTALLIZATION

Nearly all of the amino acids may readily be crystallized from aqueous solution. Although Dakin (1) has reported that he obtained β -hydroxyglutamic acid in crystalline form, other workers have usually not been so successful. This is perhaps due to its hygroscopic nature and possibly to impurities.

The crystal form varies with the solution from which the amino acid is crystallized. For purposes of decreasing the solubility, alcohol is often added to the solution of amino acid. The crystal form so obtained may not be identical with the form which is obtained when the amino acid is crystallized from aqueous solution. Amino acids, such as proline, which are very soluble in water are best crystallized from alcoholic solution. Ether may be added to decrease the solubility. Dunn (2) has obtained large crystals of some of the amino acids by permitting the solutions to evaporate very slowly.

Repeated recrystallization may be necessary for purposes of purification. In some instances the removal of traces of inorganic salts may involve considerable difficulty. Thus, Mehl and Schmidt (3) have reported that constant conductivity values were obtained in the case of synthetic glycine, alanine, and valine when these amino acids were recrystallized eight times by the addition of alcohol to the hot, saturated, aqueous solution. The crystals were washed with alcohol after each recrystallization. Recrystallization

 $K = \frac{\text{Gm. amino acid per 100 gm.} \times \text{dilution factor aqueous alcohol}}{\text{Gm. amino acid per 100 gm. water at 100}^{\circ}}.$

When these values are plotted against per cent ethyl alcohol and curves drawn through the points, the resulting graphs may be used to determine the optimum conditions under which a particular amino acid may be crystallized

¹ Dunn, Ross, and Stoddard (*J. Biol. Chem.*, 119, Proc. XXVIII, (1937)) have calculated the *crystallization constants* for a number of amino acids. This term is defined by the equation:

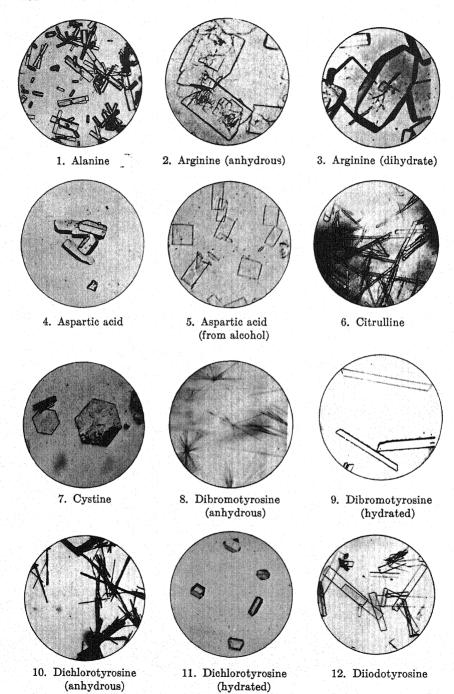


Fig. 1.

from water alone did not yield constant conductivity values even after the tenth recrystallization. Alcohol is particularly suitable for purposes of removing ammonium halides when these are the contaminating impurities. In the case of *l*-asparagine, the use of alcohol does not speed the purification, but it does reduce the loss resulting from crystallization. Thirty-five recrystallizations of

In addition to the data which are given in Table I, the following will help to characterize the amino acid crystals when viewed through the microscope.

Alanine, rods and needles; arginine, thin anhydrous plates with parallel sides and irregular ends; arginine 2H2O, rectangular prisms which frequently show pyramidal faces; aspartic acid, irregular prisms; citrulline, thin colorless prisms; cystine, clear hexagonal plates; dibromotyrosine, long needles; dibromotyrosine · ½ H2O, long transparent platelets; dichlorotyrosine, long thin rods; dichlorotyrosine · H₂O, platelets which grow into prisms; dihydroxyphenylalanine, prisms or fine needles; diiodotyrosine, long flat platelets with square ends; glutamic acid, rhombic tetrahedra; glycine, large colorless prisms, also needles; histidine (from water), thin lustrous parallel-sided plates frequently forming rosettes—various outlines; histidine (from 50 per cent alcohol), small, well-developed plates with corners cut to form an octagonal outline-aggregates form rosettes; hydroxyproline. clear platelets: \(\beta\)-hydroxyglutamic acid, thick prisms; isoleucine, fine, shiny platelets, also rods; leucine, thin, 6-sided plates and narrow rod-like plates; lysine, needles which aggregate into striated irregular plate-like forms or into radiating bunches; methionine, 6-sided plates, often massed together, not unlike leucine or phenylalanine in appearance; norleucine, shiny, 6-sided plates, grouped in clusters; phenylalanine, six-sided plates and irregular fragments; proline, thin, flat rods and needles—hygroscopic; serine, thin irregular plates; threonine, hexagonal plates not unlike serine; thyroxine, rosettes and sheaves of white needles; tryptophane, thin plates, rhombs and irregular 6-sided crystals; tyrosine, fine silky-white needles which occur in fan-shaped clusters; valine, thin, narrow, rod-shaped plates with jagged ends and long longitudinal slits.

The photomicrographs of the amino acids shown in Figs. 1, 2, and 3 are from the following sources: aspartic acid, leucine, serine, tryptophane, tyrosine, and valine (Keenan, G. L., J. Biol. Chem., 62, 163 (1924-25)); glutamic acid, histidine dichloride, phenylalanine, proline (Hawk, P. B., and Bergeim, O., Practical Physiological Chemistry, 10th edition, Philadelphia, 1931); arginine and histidine (Vickery, H. B., and Leavenworth, C. S., J. Biol. Chem., 76, 701 (1928)); lysine (Vickery, H. B., and Leavenworth, C. S., J. Biol. Chem., 76, 437 (1928)); dibromotyrosine and dichlorotyrosine (Winnek, P. S., and Schmidt, C. L. A., J. Gen. Physiol., 18, 889 (1935) and 19, 773 (1936)); citrulline (Wada, M., Biochem. Z., 224, 420 (1930)); threonine (McCoy, R. H., Meyer, C. E., and Rose, W. C., J. Biol. Chem., 112, 283 (1935-36)); alanine, cystine, glycine, hydroxyproline, isoleucine, methionine, and norleucine (kindly furnished by Dr. P. L. Kirk); thyroxine, (Kendall, E. C., and Osterberg, A. E., J. Biol. Chem., 40, 264 (1919)); homocysteine (Riegel, B., and du Vigneaud, V., J. Biol. Chem., 112, 149 (1935-36)); taurine (Plimmer, R. H. A., Practical Organic and Biochemistry, New York, Bombay, Calcutta and Madras, p. 145).

Note: For photomicrographs of the other crystal forms of d-valine see Dalton, J. B., and Schmidt, C. L. A., J. Gen. Physiol., 19, 767 (1936); and for other crystal forms of tyrosine see Andrews, J. C., J. Biol. Chem., 83, 353 (1929).

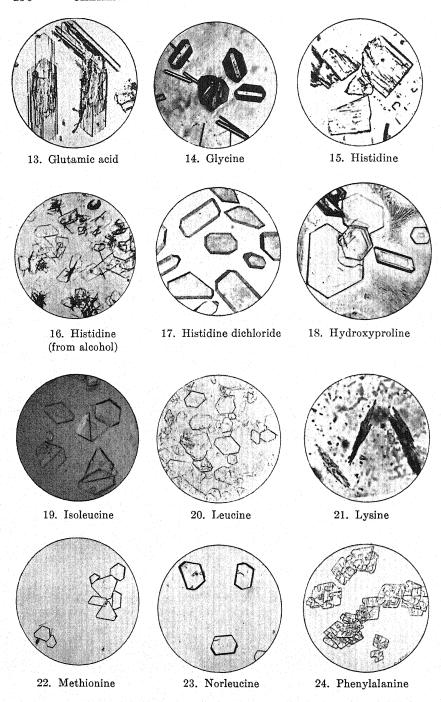


Fig. 2.

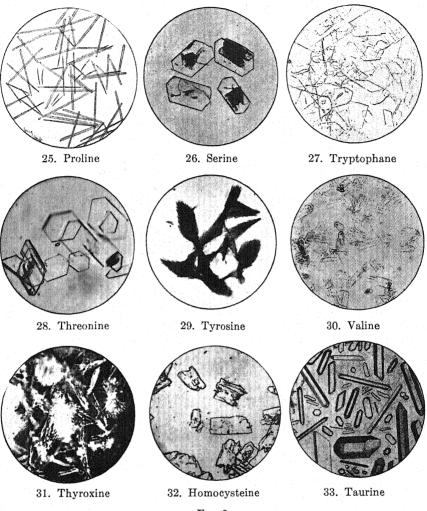


Fig. 3.

l-asparagine were found to be necessary in order to obtain constant conductivity values (3).

Purification may at times be effected by converting the amino acid into an easily recrystallizable salt. For this purpose the copper salt may at times be employed. However, a considerable number of amino acids yield well-defined copper salts, and the separation of one from several amino acids by this method may not always be successful. In the case of lysine and proline, purification may be effected by converting the amino acid into the picric acid salt and recrystallizing. In the case of arginine, flavianic acid may be so

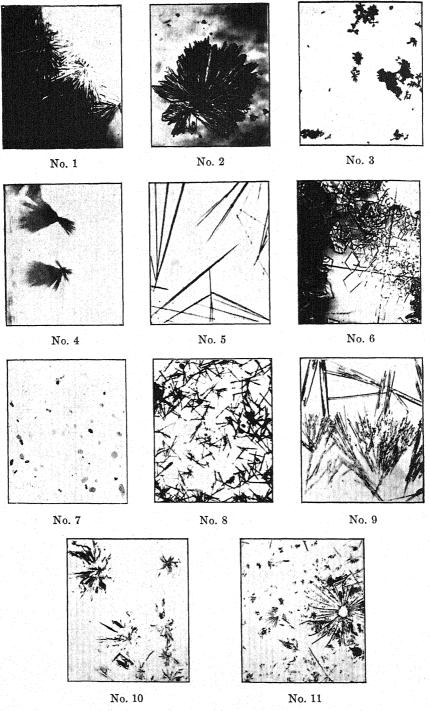


Fig. 4. Picrates of amino acids. 1. Alanine; 2. Arginine; 3. Dibromotyrosine; 4. Dichlorotyrosine; 5. Diiodotyrosine; 6. Glutamic acid; 7. Glycine; 8. Histidine; 9. Hydroxyproline; 10. Hydroxyvaline; 11. Isoleucine.

(Crosby, B. L., and Kirk, P. L., Mikrochemie, 18, 137 (1935).)

employed. The possibility that additional impurities may be introduced by the addition of reagents to solutions of amino acids as well as difficulties which may be encountered in removing traces of the reagent should always be borne in mind. The addition of reagents of the above type may materially aid in the purification of an amino acid, particularly when separation from another amino acid is desired. However, repeated recrystallization of the free amino acid is necessary if a high state of purity is desired. For purposes of physical chemical studies it may, at times, be more convenient to attempt the purification of the synthetic rather than the natural amino acid. However, the possibility that the optical forms of a racemic mixture may form a compound must be considered. Dalton and Schmidt (4) have shown that d,l-alanine, d,l-leucine, d,lisoleucine, d,l-phenylalanine, d,l-tyrosine, and d,l-aspartic acid are probably racemic compounds, while d,l-glutamic acid is probably a racemic mixture.

When the crystal properties of two amino acids are quite similar, the presence of small amounts of a contaminating amino acid may easily be overlooked. In the case of preparations of *l*-leucine, it has been shown only recently that methionine is usually present (5).

The crystal form of the amino acid may be used for purposes of identification. Photomicrographs of many of the amino acids are

CHARACTERIZATION OF PICRATES OF AMINO ACIDS

Alanine, regular rosettes of long, slender, highly refractive, deep orange-colored needles; arginine, irregular rosettes of elongated, pale yellow-colored plates; dibromotyrosine, very small sheaves and rosettes of lemon yellow-colored needles; dichlorotyrosine, sheaves and partial rosettes of slender needles; diodotyrosine, long light orange-colored needles—some tendency to form loose sheaves; glutamic acid, thin, pale yellow-colored rhombic plates-tendency to twinning to form closelypacked spheres; glycine, regular pale yellow-colored hexagonal plates—interference colors usually present; histidine, short yellow-colored rectangular needles-tendency to form small sheaves; hydroxyproline, slender, light yellow-colored, prismatic needles-marked tendency toward grouping in brushes; isoleucine, sheaves and thin rosettes of slender, light orange-colored needles; isoserine, deep yellow-colored, fern-like needles; leucine, patches of frond-like, yellow colored crystals; lysine, dull yellow-colored, elongated, somewhat irregular plates with some tendency to form loose sheaves; methionine, thin, somewhat irregular, rectangular, light yellowcolored plates; norleucine, patches of dull yellow-colored, frond-like crystals; norvaline, yellow-colored prisms with insertion twins forming crosses; phenylalanine, small, regular rosettes of short dark yellow-colored needles; proline, long, yellow-colored, hexagonal prisms; serine, short, hexagonal prisms, with marked tendency to form insertion twins; tryptophane, reddish, orange-colored plates which always cluster into spheres; valine, small, irregular, yellow-colored leaves-not very satisfactory.

Leucine, isoleucine and norleucine crystallize very slowly.

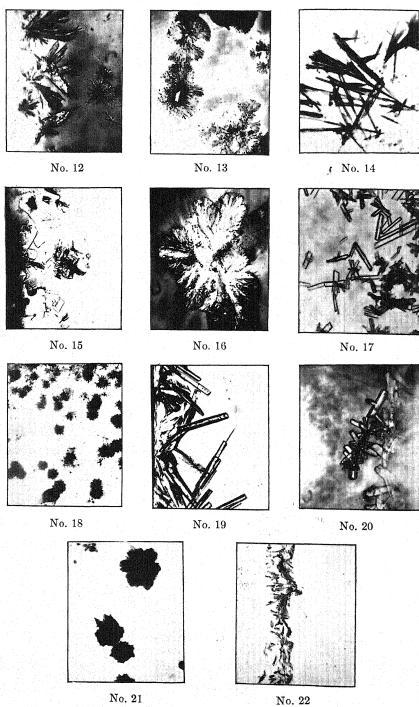


Fig. 5. Picrates of amino acids. 12. Isoserine; 13. Leucine; 14. Lysine; 15. Methionine; 16. Norleucine; 17. Norvaline; 18. Phenylalanine; 19. Proline; 20. Serine; 21. Tryptophane; 22. Valine.

(Crosby, B. L., and Kirk, P. L., Mikrochemie, 18, 137 (1935).)

Optical Features of Amino Acids¹ TABLE I

	Arginine lanine (dihy- drate)	rginine Arginine dihy- (anhy- lrate) drous)	Aspartic Cystine	Cystine	Glu- tamic acid	Glycine	Histi-	Leu-	Leu- Phenyl- Serine Trypto- Tyro-	Serine	Trypto. phane	Tyro-	Valine
	Needles Irregular frag- ments	Plates	Plates	Prisms	Plates	Plates	Plates	Plates	Plates	Plates	Plates	Plates Needles	Plates
-	515 1 528	1.548	515	1.640	1.490	1.495	1.520	1.525	1.600	1.515	1.625	1.550	1.495
- - ∓			1.560 1.700	1.700	1.605	1.615	Indeter-	1.535	1.615 Indeter 1.535 1.610 1.575	1.575	€)	1.600	(3)
							minate						
~	1.575 1.579	1.610	1.630	1.700	1.620	1.650	1.610	1.560	1.650 1.610 1.560 1.675	1.586		1.680	1.565
9	$0 \mid 0.051$	0.062	0.115	090.0	0.130	0.155	0.090	0.035	0.075	0.071		0.130	0.070
~	1.575 1.579	1.562	1.630	1.700	1.605	1.615	1.610	1.535	1.535 1.675	-	1.635	1.680	1.565
$^{\circ}$	1-2 1-2	1-2	3-4		2-3	1-2	1-2		1-2	2-3		-	
	Extinction Parallel		£	Indeter-	(3)	(;)	Straight Parallel Inclined	Parallel	Inclined	£	<u></u>	Parallel Parallel	Parallel
				minate	,				Ξ,				
			Indeter-	Indeter-	Indeter- Indeter- Indeter-	Indeter-	1	Indeter-	Indeter-Indeter-Indeter-Indeter-	Indeter-	Indeter-	+	+
			minate	minate	minate minate minate minate	minate		minate	minate minate minate minate	minate	minate		

For optical features of (a) hydroxyproline, see Fischer, E., Ber. Chem. Ges., 35, 2660 (1902).
 (b) alanine, see Fischer, E., Ber. Chem. Ges., 39, 453 (1906).
 (Keenan, G. L., J. Biol. Chem., 62, 163, 1924-25; 83, 137, 1929.)

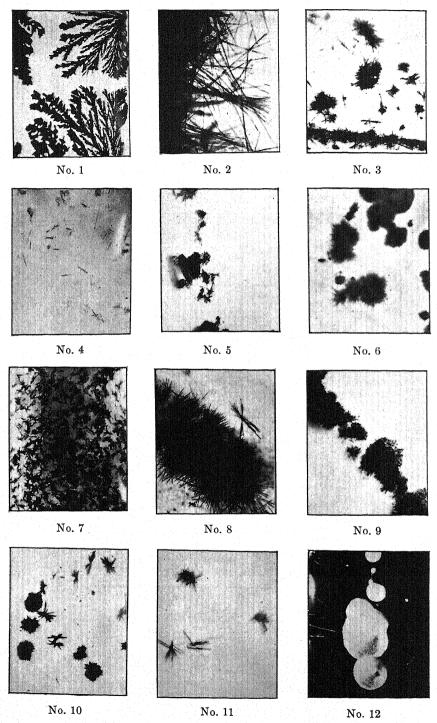


Fig. 6. Flavianates of amino acids. 1. Arginine; 2. Aspartic acid; 3. Dichlorotyrosine; 4. Glutamic acid; 5. Histidine; 6. Isoleucine; 7. Leucine; 8. Methionine; 9. Norleucine; 10. Tryptophane; 11. Tyrosine; 12. Valine.

(Crosby, B. L., and Kirk, P. L., Mikrochemie, 18, 137 (1935).)

given in Figs. 1 to 3. At times the crystal form of the amino acid may not assume the usual characteristic appearances. Thus, Andrews (6) has reported having obtained l-tyrosine as blunt prisms resembling somewhat the crystals of aspartic acid, and not the silk-like needles which are usually observed. However, the optical properties of the prisms were found to be identical with those of the usual form of l-tyrosine. Dalton and Schmidt (7) have shown that d-valine may exist in several crystalline forms, and that the solubility of this amino acid varies with the crystal form. Certain amino acids may include water of crystallization in the molecule. Thus, Winnek and Schmidt (8) have obtained both the hydrated and anhydrous forms of l-dichloro- and dibromotyrosine, and Vickery and Leavenworth (9) have prepared arginine dihydrate.

Keenan and others (10) have reported crystallographic data on many of the amino acids. The data are given in Table I. Such measurements, in the hands of experienced workers, afford a ready method of identifying amino acids, especially in those cases in which convenient chemical methods are not available.

CHARACTERIZATION OF FLAVIANATES OF AMINO ACIDS

Arginine, thin elongated plates—end proliferation of crystals caused formation of moss-like clusters, anisotropic, elongation (+), parallel extinction, Np>1.778, moderate birefringence, Ng about 1.85; aspartic acid, slender yellow-colored needles in loose rosettes; dichlorotyrosine, loose rosettes of fibrous yellow-colored crystals, anisotropic, elongation (-), parallel extinction, Np 1.458, Ng about 1.75; diiodotyrosine, small loose rosettes of fibrous yellow-colored crystals, anisotropic, elongation (-), parallel extinction, Np 1.495, Ng 1.720; glutamic acid, very thin, elongated, pale yellow-colored hexagonal plates; histidine, short lemon yellowcolored needles in small rosettes, anisotropic, extinction 18°, Np 1.546, Ng 1.735; isoleucine, rosettes and ropes of slender, rather fibrous yellow-colored needles, anisotropic, elongation (-), parallel extinction, Np 1.616, Ng 1.666; leucine, very small rosettes of slender yellow-colored needles, anisotropic, elongation (+), parallel extinction, Np 1.60, Ng 1.651; lysine, rosettes of slender, almost fibrous, yellowcolored needles, anisotropic, elongation (-), parallel extinction, Np 1.546, Ng 1.735; methionine, sheaves and loose rosettes of yellow-colored needles, anisotropic, elongation (-), parallel extinction, Np 1.635, Ng 1.683; norleucine, short yellowcolored needles in closely-packed rosettes; tryptophane, rosettes of short yellowcolored needles, anisotropic, elongation (-), parallel extinction, Np 1.493, Ng 1.778; tyrosine, elongated, well-defined, bright yellow-colored prisms—tendency to cluster into spheres, anisotropic, elongation (-), parallel extinction, Np 1.493, Ng 1.778; valine, pale yellow-colored needles, growing in flat closely-packed eccentric rosettes, giving a shell-like appearance.

Alanine, glycine, cystine, dibromotyrosine, proline, hydroxyproline, phenylalanine, and serine did not yield crystals.

Np = the index for fast ray; Ng = the index for slow ray.

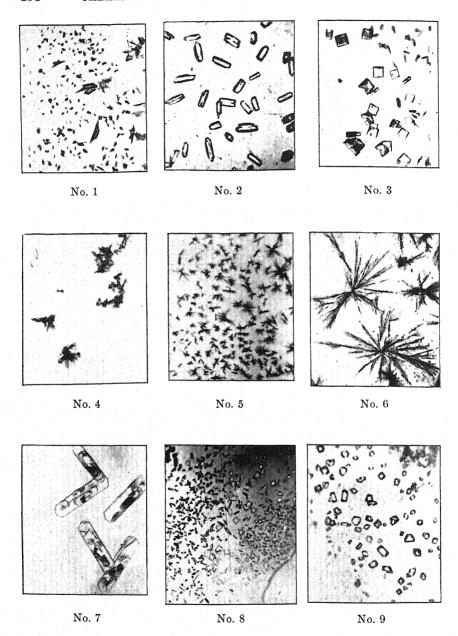


Fig. 7. Phosphotungstates of amino acids. 1. Alanine (early stage); 2. Alanine (later stage); 3. Glycine; 4. Lysine; 5. Arginine; 6. Cystine; 7. Isoserine; 8. Serine: 9. Proline.

(Bullock, B., and Kirk, P. L., Mikrochemie, 18, 129 (1935).)

Kirk and his co-workers (11) have reported several of their systematic studies on the crystal properties of the readily crystallizable salts of amino acids. Certain of their photomicrographs are reproduced in Figs. 4 to 8. These data should prove very useful for purposes of identifying amino acids.

Many of the proteins have also been obtained in crystalline form (see Fig. 9). This is especially true of hemoglobin and the vegetable proteins. In general, the animal proteins are more difficult to crystallize than the vegetable proteins. Urease (12) (Fig. 10) and certain of the proteolytic enzymes (13) (Figs. 11 to 14) have been obtained as crystalline products. Since certain of the filtrable viruses appear to be crystallizable (14), it will be of great interest if these substances prove to be protein in nature. Protein crystals are optically true crystals. It is altogether probable that the same atomic forces act in the compounds which have large molecular weights as in those with smaller molecules (15).

Hemoglobin especially has been studied from the standpoint of crystalline form. Reichert and Brown (16) have carried out extensive studies in this field. No attempt was made, however, to obtain pure preparations of hemoglobin. It is possible that the various proteins which are present in the red cell may have considerable effect on the crystal habit and even the crystal system and form. This statement finds support in the fact observed by Halliburton (17) and confirmed by Reichert (18) that the crystal-line form of oxyhemoglobin derived from a given species may be

CHARACTERIZATION OF PHOSPHOTUNGSTATES OF AMINO ACIDS

Alanine, prisms having pointed or rounded ends, often with the appearance of a partial lengthwise split, anisotropic with parallel extinction and elongation (—); arginine, clusters of needles developing plate-like ends, anisotropic with parallel extinction; cystine, clusters of needles, developing plate-like ends, often pointed, anisotropic with parallel extinction and elongation (+); glycine, square thin isotropic plates and rectangular plates, anisotropic with parallel extinction and elongation (+); histidine, small radial clusters of needles similar to arginine phosphotung-state developing into plates which are often rectangular, anisotropic with parallel extinction and elongation (+); hydroxyproline, like that of proline; isoserine, six-sided plates having small similar plates developed on the flat surface; lysine, radial clusters of needles developing into clustered rectangular plates, anisotropic with parallel extinction; proline, white precipitate or oily drops followed by highly refractive, many faced crystals, anisotropic with parallel extinction showing a variety of interference colors, elongation indeterminable; serine, square flat plates and pointed irregular prisms, isotropic.

¹ See also, Toennies, G., and Elliott, M., J. Biol. Chem., 111, 61 (1935) for photomicrograph.

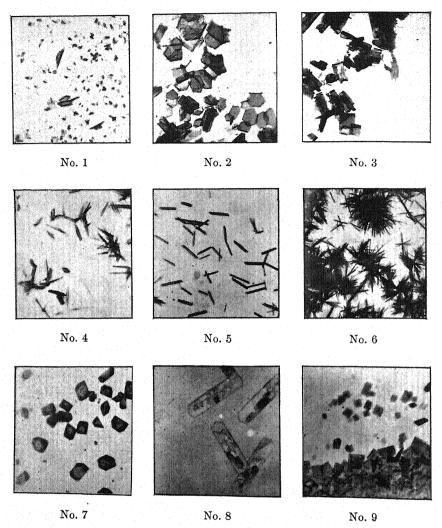


Fig. 8. Phosphomolybdates of amino acids. 1. Alanine (early stage); 2. Alanine (later stage); 3. Glycine; 4. Arginine; 5. Histidine; 6. Lysine; 7. Proline; 8. Isoserine; 9. Serine.

(Bullock, B., and Kirk, P. L., Mikrochemie, 18, 129 (1935).)

profoundly modified by admixture with the blood of another species. According to Reichert the degree of modification of the crystalline form resulting from admixture of two bloods depends very greatly upon the proportion in which they are mixed. According to Wichmann (19) and Katz (20), crystalline proteins swell in or absorb the surrounding fluid menstruum in a manner analogous to the swelling of jellies. Repeated recrystallization of

the protein is therefore necessary in order to remove completely traces of substances which may have been present in the original menstruum in which crystallization took place. According to Schulze and Zsigmondy (21), egg albumin must be recrystallized three to six times in order to remove appreciable contamination by other proteins. It is indeed possible that the antigenic properties of proteins may be profoundly influenced by traces of contaminating proteins.

In more recent times, Boor (22) has prepared hemoglobin crysstals from the blood of various animals. It is evident from Fig. 9 that the crystal form of hemoglobin varies with different animals. There are even differences in the crystalline forms of oxyhemoglobin and carbon monoxide hemoglobin from the same animal.

Whether hemoglobins from different animals are chemically identical or not cannot be decided on the basis of crystalline form alone. According to Hüfner (23), Butterfield (24), Heubner and Rosenberg (25), and Schumm (26), the characteristic absorption bands and the ratio of the absorption of light in different parts of the spectrum of hemoglobin are identical in such species as the horse and man (Schumm), and the rabbit, sheep, and hog (Heubner and Rosenberg). If internal differences of atomic arrangement are present they should manifest themselves in these properties. Differences in chemical structure of proteins can be brought out by immunological methods. Hemoglobin is weakly antigenic. According to Heidelberger and Landsteiner (27), hemoglobin gives a species-specific precipitin reaction, although hemoglobins of some other species will cross react, but to a much lesser degree. No

CHARACTERIZATION OF PHOSPHOMOLYBDATES OF AMINO ACIDS

Alanine, irregular edged prisms, pointed and curved and often paired—these developed into six-sided prisms with occasional irregular edges and imperfect shapes, anisotropic with parallel extinction; arginine, two types—loose clusters of rectangular plates, anisotropic with parallel extinction and elongation (+) and some highly refractive prismatic crystals, anisotropic with parallel extinction; cystine, similar to those of lysine, elongation (+); glycine, rectangular and plate-like, grouping near edge of drop, anisotropic with parallel extinction; histidine, radial clusters of needles developing into rectangular plates, anisotropic with parallel extinction; hydroxyproline, similar to that of proline; isoserine, elongated, six-sided plates with smaller plates attached to the flat surfaces; lysine, radial clusters of needles and isolated needles developing plate-like ends, anisotropic with parallel extinction; proline, highly refractive, multiple faced crystals, anisotropic with parallel extinction—also some isotropic prismatic forms; serine, flat, square, isotropic plates with low refractive index, form on edge of drop.

differences were found between oxyhemoglobin, carbon monoxide hemoglobin, and cyanhemoglobin from the same species. It would be desirable to have extensive studies of the crystal structure of

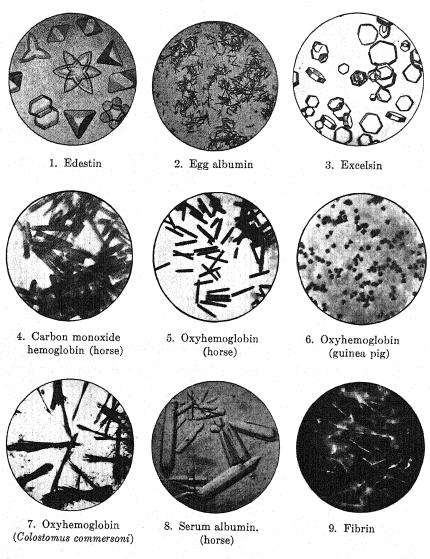


Fig. 9.

hemoglobin from different species carried out by the X-ray method (see next section). According to Haurowitz, Winkler and Kraus (28), three distinct hemoglobins occur in man: one predominating in the adult, another in the fetus, and a third in the muscles.

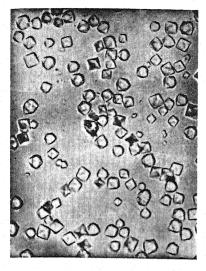
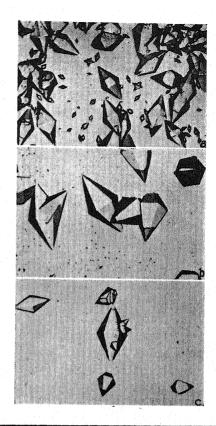


Fig. 10. Photomicrograph of urease crystals magnified 728 diameters. (Sumner, J. B., J. Biol. Chem., 69, 435 (1926).)

Fig. 11. Crystalline pepsin, (a); and crystalline acetylated pepsin (b and c). (Herriott, R. M., and Northrop, J. H., J. Gen. Physiol., 18, 35 (1934).)



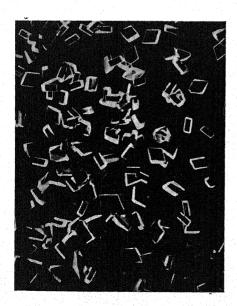


Fig. 12. Crystals of chymo-trypsin. (Kunitz, M., and Northrop, J. H., J. Gen. Physiol., 18, 433 (1935).)

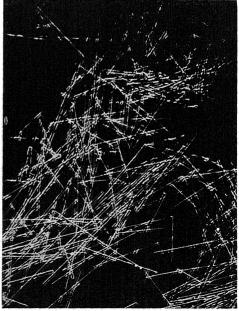


Fig. 13. Crystals of chymo-trypsinogen

Chymo-trypsinogen is activated by trypsin, not by enterokinase. (Kunitz, M., and Northrop, J. H., J. Gen. Physiol., 18, 433 (1935).)

As has already been stated, certain proteins, particularly the vegetable proteins and hemoglobin, can readily be obtained in the form of crystals. The ease with which hemoglobin will crystallize varies with the species from which it is derived. Horse and dog hemoglobin is quite easily crystallizable, while beef hemoglobin can be crystallized only with great difficulty. Other proteins, such as ovalbumin and serum albumin, yield crystals only with considerable difficulty. In order to crystallize these proteins, it is necessary that inorganic salts in very high concentration be present (29, 30, 31, 32). The coagulum which is formed becomes crystalline on standing. It is quite probable that under such conditions the crystalline protein is not in the isoelectric condition.

The function of ammonium sulfate has been regarded as that of a dehydrating agent. In other words, the implication is that there is greater tendency of water to associate itself with the salt than with the hydrocarbon chain or the amino and carboxyl dipoles of the protein molecule. Chick and Martin (33) investigated the composition of the precipitate of egg albumin after subjecting it to a pressure of three tons per square inch in order to remove as much of the mother liquor as possible. The content of ammonium sulfate varied between 30 and 22 per cent, and that of water between 8 and 6 per cent. The amount of water in the protein precipitate is evidently determined by the content of ammonium sulfate.

According to Sörensen (34), crystalline ovalbumin is obtained not at pH 4.8, the isoelectric point, but at pH 4.58. This indicates that the crystals consist of ovalbumin sulfate. Sörensen washed the crystalline material with sodium chloride solution until the washings no longer gave a test for sulfates. He was unwilling to draw any conclusions as to whether or not the crystalline protein contained ammonium sulfate. The greater part of the ovalbumin dissolved in water. It is possible that some hydrolysis of the protein sulfate takes place in aqueous solution. The amount of uncombined protein so formed must, however, be very small. It appears safe, therefore, to conclude that the crystalline material is a single entity. According to Wichmann (19) all albumin crystals are either crystallographically identical or else isomorphic.

The addition of small amounts of alcohol facilitates crystallization of hemoglobin (32, 36, 37). The amount which is necessary is, however, insufficient to lead to any appreciable amount of denaturation. The function of alcohol is to decrease the solubility of the protein. If, prior to addition of alcohol, the aqueous solution is

not brought to the isoelectric point of the protein, the crystals so obtained represent the protein salt and not the free protein.

Howell (35) has shown that fibrin is a crystalline protein. It is obtained by precipitating fibrinogen with thrombin. In the

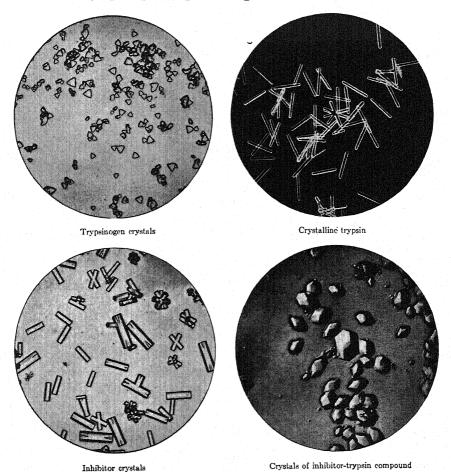


Fig. 14.

(Kunitz, M., and Northrop, J. H., J. Gen. Physiol., 19, 991 (1936); For crystallization of pepsinogen, see Herriott, R. M., and Northrop, J. H., Science, 83, 469 1936); Herriott, R. M., J. Gen. Physiol., 21, 501 (1938).)

presence of hydrogen ions fibrin separates out in the form of crystalline needles.

2. CRYSTAL STRUCTURE

Reference has been made in Chapter VII to X-ray patterns of proteins. The object of carrying out studies on the crystal structure

of amino acids and proteins is to obtain information as to the organization of the substance in the solid state, particularly the dimensions and shape of the unit crystal cell or, in other words, the arrangement of the molecules within the crystal and the arrangement of the atoms within the molecule. In the case of proteins which do not have a definite crystalline structure there is probably a less definite arrangement of the atoms than in simple crystals

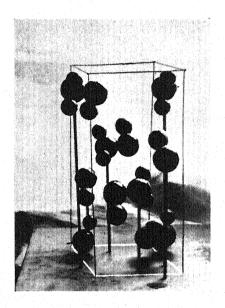


Fig. 15. Model of glycine.

Large balls = carbon,

Medium balls = nitrogen,

Small balls = oxygen.

(Hengstenberg, J., and Lenel, F. V., Z. Kristallographie, 77, 424 (1931).)

and hence difficulties in obtaining a picture of atomic arrangement present themselves. It might be expected that the molecules of which the crystal is composed have the same size and shape as they have when present in solution except so far as they may be modified by crystal forces; this factor, however, is probably of minor influence. Complete data on crystal structure should yield valuable information on stereochemical problems under which subject we may list optical rotation.

Up to the present time complete crystal structures are known for a comparatively small number of organic compounds and these are not of biochemical interest. However, as has been indicated in Chapter VII, such partial information may form the basis of generalized concepts of molecular structure, particularly in the case of fibrous structures such as silk and wool.

Complete crystal analysis of amino acids is essential to an understanding of the structure of protein molecules. Although a number

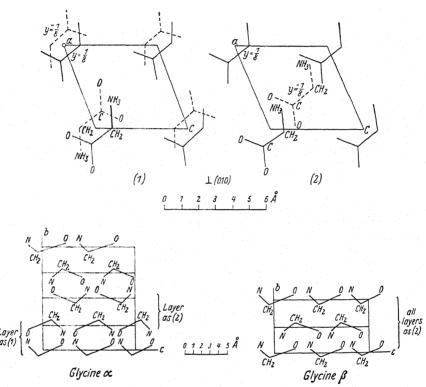


Fig. 16. The crystal structure of glycine. (Bernal, J. D., Z. Kristallographie, 78, 363 (1931).)

of investigations on crystal analysis of amino acids have been reported, none of them can be considered complete. This is in part due to the fact that in amino acids we are dealing with atoms which are very nearly alike, viz., carbon, oxygen, and nitrogen.

Hengstenberg and Lenel (38) used a modification of the method which Bragg and West (39) employed with silicates for the analysis of the crystal structure of glycine. The method is based upon a determination of the variation in electron density throughout the crystal. The regions of greatest density are those in the immediate vicinity of the atoms. When these are located, the positions of the atoms are determined. When it is necessary to deal with atoms

which are quite alike, the method is less accurate than otherwise. A model showing the arrangement of the atoms in glycine is shown in Fig. 15. The general form of the glycine molecule is similar to a model constructed from the accepted dimensions of atomic radii and their tetrahedral directional valences. The assigned space

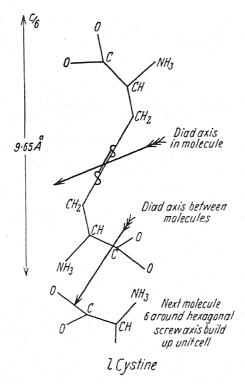


Fig. 17. The crystal structure of cystine. (Bernal, J. D., Z. Kristallographie, 78, 363 (1931).)

group requires a paired arrangement of molecules in the crystal such as might result from pairing zwitterions.

Bernal (40) has reported a preliminary study of the crystal structures of fifteen compounds, most of which are amino acids, and the remainder are peptides. His work indicates that the molecules pack together in double molecules or in extended chains with the dipole group as the determining factor. Two crystal forms of glycine were studied. One form was obtained by crystallizing the amino acid from water, the other (needles) was obtained by adding alcohol to the aqueous solution of the amino acid. The structure of the two forms of glycine is shown in Fig. 16. The

 α -amino groups of glycine pack together in double sheets so that every NH₃-group is surrounded by six oxygen atoms in the plane of the molecule and by two other pairs above and below. The two forms differ in that when glycine is crystallized from water, there is, in addition, a packing of two amino groups by a center of symmetry into a double molecule.

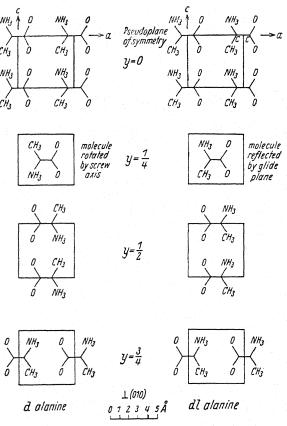


Fig. 18. The crystal structure of alanine. (Bernal, J. D., Z. Kristallographie, 78, 363 (1931).)

In cystine six asymmetric sheets composed of cystine chains which are packed side by side pile together to form the hexagonal unit cell (Fig. 17). Alanine differs from these by the addition of a methyl group which turns the triangular group,

$$H_2$$
 C C C

into a quadrangular group,

Diketopiperazine

$$CH_3$$
 O $C-C$ H_3N O

The active and inactive forms differ only very slightly from each other since, due to the pseudo plane of symmetry in the molecule,

Fig. 19. The crystal structure of various compounds. (Bernal, J. D., Z. Kristallographie, 78, 363 (1931).)

Glycylqlycine

both are approximately holohedral crystals, as shown in Fig. 18. This probably accounts for the small optical rotation of alanine.

In the case of aspartic acid, two molecules are associated into an asymmetric unit through the terminal carboxyl groups. Similar associated pairs occur in phenylalanine. The large phenyl group gives the molecule its low density and softness, properties which are characteristic of aromatic rather than amino acid compounds. In the case of aspartic acid, the carbon chain is probably straight due to its shortness, while the form of the cell and the properties of asparagine and of glutamic acid indicate the existence of unassociated ring-like molecules. Glycine anhydride is built from centro-symmetrical, almost flat, hexagonal molecules linked together in ribbons by their residual electrical forces. The three forms of

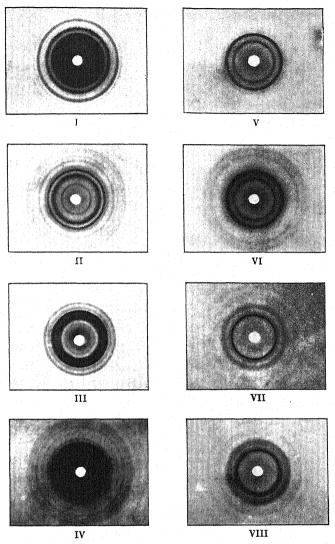


Fig. 20. X-ray patterns of glycine and glycine peptides. I Glycine, II Glycyl-glycine, III Diglycyl-glycine, IV Triglycyl-glycine, V Tetra-, VI Penta-, VII Hexa-, VIII Hepta-glycyl-glycine.

(Meyer, K. H., and Go, Y., Helv. Chim. Acta, 17, 1488 (1934).)

glycylglycine and the one diglycylglycine form show chains of a "trans" character packed in different ways. The schematic arrangement of the atoms in the above mentioned compounds is shown in Fig. 19.

X-ray patterns of glycine and glycine-peptides which were obtained by Meyer and Go (41) are represented in Fig. 20. These are similar to crystal-powder diagrams. Interpretations from such diagrams cannot be made accurately without crystallographic data. The diagrams indicate a simple arrangement of chains in planes which are spaced 4.15 Å units apart.

In the case of proteins such as wool, silk, and hair, the long chain molecules are parallel and the units of which the chain is composed lose a great deal of their individuality. The X-ray diagrams consist of a regular arrangement of spots which may be drawn out into arcs. Investigation in this field has shown that a distance of about 3.5 Å units is associated with the long axis of the fiber, especially

[N.B. The side-chains (R-groups) must be thought of as standing out above and below the plane of the paper.]

Fig. 21. Schematic structure of the protein molecule. (Astbury, W. T., and Lomax, R., J. Chem. Soc. London, 1935, Part I, p. 846.)

when it is stretched to nearly its elastic limit. This has been interpreted as indicating the length of the peptide group in the chain since it checks well with a model built with the accepted atomic radii and the tetrahedral angles of carbon and nitrogen. We may consider that the protein molecule consists of a backbone of peptide groups containing the R-radicals of the different amino acids as is indicated by the type formula which is given in Fig. 21. Dimensions of 4.5 Å units, which are about the same in all proteins, and 10.5 Å units, which occur in many proteins but may vary in others, have been found in the cross sections of the fiber. Both dimensions are considered to be lateral distances which separate the chain molecules. The zigzag backbones with the side chains form layers which are separated by 4.5 Å units, whereas the long parallel-lying molecules are separated 10.5 Å units apart by the side chains. Detailed accounts of this subject are given by Astbury and his co-workers (42).

Only a few investigations on the crystal forms of proteins have

been published. Bernal and Crowfoot (43) have made a preliminary study of crystalline pepsin. They consider that the protein molecules are relatively dense globular bodies, possibly joined together by valency bridges, and separated by relatively large spaces which contain water. They believe it possible that peptide chains may exist only in the fibrous proteins, while the molecules of the soluble proteins may have their constituent parts grouped more symmetrically around a prosthetic nucleus. Their data suggest that Svedberg's idea that proteins are built up from unit molecules has fundamental crystallographic significance (44).

Wyckhoff and Corey (45) have reported some preliminary studies on the crystal structure of edestin, excelsin, and hemoglobin. These proteins, like pepsin, contain water of crystallization which is rapidly lost when the crystals are dried. The apparently amorphous materials resulting from the efflorescence of the crystalline compounds yield band-patterns which are common to other proteins as well. Data obtained by these workers are given in Table II.

Table II

Spacings of Some Powder Lines of Rat Oxyhemoglobin
(Obtained with the chromium K radiation on moist crystals)

Spacing Å	Estimated intensity	Spacing Å	Estimated intensity
45.7	8	10.4	m
27.5	m	9.4	f
21.7	m-	8.4	f
18.0	ff	5.93	ff
15.4	f	4.90	ff
13.0	m	4.62	f
11.6	ff	3.47	ff

s, m, f, ff represent strong, medium, faint, and very faint. (Wyckoff, R. W. G., and Corey, R. B., Science, 81, 365, 1935.)

It is evident from the above discussion that a great deal of work will have to be carried out before our knowledge of this subject can be considered adequate. Even in the case of amino acids data obtained by different workers do not agree within the desired limits of accuracy. When adequate techniques become available, the study of crystal structure of amino acids and proteins should do much towards elucidating the chemical configuration of proteins.

3. DENSITIES OF PROTEIN CRYSTALS

Comparatively few studies on the densities of protein crystals have been carried out. Eyer (46) gives the value 1.315 for insulin, while Crowfoot (47) finds 1.306. Bernal and Crowfoot (43) assign a value of 1.28 for the density of pepsin, and Adair and Adair (48) find 1.296 for hemoglobin.

More recently Adair and Adair (49) have again carried out studies in this field. The technique employed consists in observing the flotation of protein crystals, on centrifuging, in media of different densities. Organic liquids cannot be used for this purpose since the protein crystals cannot be freed from the film of aqueous media without possibly producing irreversible changes. When aqueous media are employed they must have a high density and they must not dissolve or denature the protein crystals. To determine whether or not the media produced any changes in the composition of a particular protein crystal, the densities were determined in different media, and, if identical results were not obtained, the reversibility of the effects produced by a given medium was tested by comparing the densities of the crystal before and after treatment by the medium.

A variety of aqueous media were employed. They contained such compounds as sucrose, ammonium sulfate, sodium phosphate, and potassium citrate. It was found preferable to use mixtures rather than solutions of single salts. In certain of the media the pH was varied in order to study the effects of this factor on the density of the protein crystal.

The calculation of the density is made as follows: if D_s = the density of the more concentrated solution in the series where sedimentation takes place, and D_f = the density of the least dense solution where no sediment of crystals was noted, the true density should be intermediate between D_s and D_f . The mean value, D_s , is equal to one-half of the sum of these two quantities, and the difference, D^{\pm} , is equal to $D - D_s$ or $D_f - D$. Table III gives the data obtained on various hemoglobins.

While it is possible to reproduce data obtained with a given medium, the densities of protein crystals vary with different media as well as with the pH of the solution. The density of crystals of sheep hemoglobin suspended in a solution of sodium phosphate was found to be 1.2269, while, in a solution which contained sucrose and ammonium sulfate, the density was 1.2642. The density reverted to the original value when the crystals were washed and resus-

Table III Density of Crystals of Hemoglobin

Medium AB half-saturated ammonium sulfate+sucrose. Medium CD saturated ammonium sulfate+sucrose. Medium EF saturated ammonium sulfate+sucrose, pH 6.8.

Species	\mathbf{Medium}	Temp. (degrees)	Density	Difference	
Horse	AB	19.0	1.2699	0.0001	
Horse	AB	19.9	1.2700	0.0040	
Horse	AB	20.0	1.2700	0.0020	
Sheep	CD	22.0	1.2696	0.0010	
Sheep		20.8	1.2669	0.0027	
Sheep		19.8	1.2642	0.0010	
Horse	EF	17.9	1.2656	0.0016	

(Adair, G. S., and Adair, M. E., Proc. Roy. Soc. London, 120 B, 422 (1936).)

pended in the solution of sodium phosphate. The results may be explained on the basis that protein crystals are solid phases containing protein rather than being a pure substance, and that the constituents of the media can diffuse into the protein-containing phase. The composition of this phase and its density are a function of the composition of the liquid. The density of heat coagulated proteins is only slightly greater than that of the corresponding protein crystals. Edestin was found to have a higher crystal density than serum albumin, egg albumin, egg globulin, and hemoglobin.

A comparison between the densities of the protein crystals and the apparent specific volumes of the proteins in solution may be made provided that the latter quantity be determined in the same types of aqueous solutions as are used for the estimation of crystal densities. The partial specific volume of the dry protein, v_p , may be defined as the increase in the volume of solution caused by the addition of one gm. of dry protein. It is given by the equation,

$$v_p = (\partial V/\partial m_p) \tag{1}$$

where V = volume of solution, and m_p = mass of anhydrous protein. It is assumed that the pressure, temperature, and masses of all of the substances, except the protein, are constant. The partial specific volumes of some proteins are given in Table IV. The density values which are starred are the reciprocals of the partial specific volumes. Although the densities of different proteins in aqueous solutions vary somewhat from each other, they lie in the

vicinity of 1.34, a value which is decidedly larger than that of the densities of protein crystals.

Table IV

Partial Specific Volumes of Proteins

Protein	Temp. (degrees)	Volume	Density	Medium
	(a)	In aqueo	us solutio	n
Sheep hemoglobin	0	0.7431	1.3457	The state of the s
Sheep hemoglobin	16	0.7506	1.3322	
Sheep hemoglobin	20	0.7524	1.3290*	
Horse serum albumin.	20	0.7418	1.3481*	
Egg albumin	20	0.7457	1.3410	
Water	20	1.0018	0.9982*	
		T 1:00	,	
Edestin ¹	1		ent media	
	20	0.744	1.346*	1.24 M NaCl, pH 5.5
Edestin	20 20		<u> </u>	
Edestin ¹ Edestin Egg albumin ² Egg albumin	20 20	0.744 0.745	1.346* 1.342	1.24 M NaCl, pH 5.5 0.62 M NaCl, pH 11.3 Water
Edestin	20 20 20 20 20	0.744 0.745 0.749	1.346* 1.342 1.335	1.24 M NaCl, pH 5.5 0.62 M NaCl, pH 11.3 Water 0.017 M phosphate, pH 7.
Edestin	20 20 20 20 20 20 20	0.744 0.745 0.749 0.744	1.346* 1.342 1.335 1.344	1.24 M NaCl, pH 5.5 0.62 M NaCl, pH 11.3 Water
Edestin	20 20 20 20 20 20 20 20	0.744 0.745 0.749 0.744 0.749	1.346* 1.342 1.335 1.344 1.335	1.24 M NaCl, pH 5.5 0.62 M NaCl, pH 11.3 Water 0.017 M phosphate, pH 7. 0.02 M acetate, pH 4.7
Edestin	20 20 20 20 20 20 20 20 20	0.744 0.745 0.749 0.744 0.749 0.744	1.346* 1.342 1.335 1.344 1.335 1.344*	1.24 M NaCl, pH 5.5 0.62 M NaCl, pH 11.3 Water 0.017 M phosphate, pH 7. 0.02 M acetate, pH 4.7
Edestin	20 20 20 20 20 20 20 20 20	0.744 0.745 0.749 0.744 0.749 0.744 0.745	1.346* 1.342 1.335 1.344 1.335 1.344* 1.342	1.24 M NaCl, pH 5.5 0.62 M NaCl, pH 11.3 Water 0.017 M phosphate, pH 7. 0.02 M acetate, pH 4.7

¹ Svedberg, T., and Stamm, A. J., J. Amer. Chem. Soc., 51, 2170 (1929).

² Nichols, J. B., J. Amer. Chem. Soc., 52, 5176 (1930).

* Calculated according to equation (8) in Adair's paper.

(Adair, G. S., and Adair, M. E., Proc. Roy. Soc. London, 120 B, 422 (1936).)

The above experiments are of interest in that they have a bearing on the subject of the hydration of protein crystals. We may consider the protein crystal as being made up of two components, A and B, where A is a mixture of protein and water, and B is a mixture of water and salt in the proportions as they exist in the dispersing medium. Sörensen (34) has used the following formula to calculate the hydration of egg albumin crystals which were equilibrated with ammonium sulfate:

$$w = (r - r_a)/r_a \tag{2}$$

where w = apparent hydration in gm. of water per gm. of anhydrous protein, r = gm. protein hydrate per gm. protein nitrogen, and

³ Svedberg, T., and Sjögren, B., J. Amer. Chem. Soc., 50, 3318 (1928).

⁴ Svedberg, T., and Nichols, J. B., J. Amer. Chem. Soc., 49, 2920 (1927).

 $r_a = \text{gm.}$ anhydrous protein per gm. protein nitrogen. Values for r are computed from measurements of ammonia and protein nitrogen in the filtrate. Adair and Adair have calculated values for w which are based on data which are found in the literature. These are given in Table V.

Table V

Hydration of Crystalline, Precipitated, and Coagulated Proteins
by Sörensen's Method of Proportionality

Protein	\mathbf{Medium}	pН	r	r_a	w
Egg albumin crystals	Ammonium phosphate	4.8	7.891	6.4^{7}	0.233
Egg albumin crystals	Ammonium phosphate	5.5	8.12^{1}	6.4^{7}	0.269
Egg albumin crystals	Ammonium sulfate		7.86^{2}	6.4^{7}	0.228^{2}
Egg albumin coagulated	Ammonium sulfate		7.65^{3}	6.4^{7}	0.195
Egg albumin coagulated	Sucrose		7.49^{3}	6.4^{7}	0.171
Serum albumin crystals	Ammonium sulfate		8.354	6.418	0.303
Pseudo-globulin precipitated	Ammonium sulfate		8.884	6.618	0.343
Globulin (ox)	Ammonium sulfate		8.745		
Albumin (ox)	Ammonium sulfate		8.315		
Horse hemoglobin crystals	Ammonium sulfate		7.82^{6}	5.999	0.306

¹ Sörensen, S. P. L., and Palitzsch, S., Compt. rend. Lab. Carlsberg, 15, No. 2 (1923).

² Sörensen, S. P. L., Compt. rend. Lab. Carlsberg, 12, 164 (1917).

⁴ Sörensen, S. P. L., Compt. rend. Lab. Carlsberg, 15, No. 11 (1925).

Bonot, A., Thèse Doct. Sci. Paris, 1934.

⁷ Taylor, G. L., Adair, G. S., and Adair, M. E., J. Hyg., 32, 340 (1932).

⁸ Adair, G. S., and Robinson, M. E., Biochem. J., 24, 993 (1930).

Vickery, H. B., and Leavenworth, C. S., J. Biol. Chem., 79, 377 (1928).
 (Adair, G. S., and Adair, M. E., Proc. Roy. Soc. London, 120 B, 422 (1936).)

Values for w may also be calculated in the following manner. If x = weight fraction of water, and 1 - x = weight fraction of anhydrous protein, the specific volume of component A is given by

$$v_{A} = (1 - x)v_{p} + xv_{1} \tag{3}$$

where v_A = specific volume of component A which contains w gm. water per gm. dry protein, v_p = partial specific volume of anhydrous protein, and v_1 = specific volume of water. If we assume w = x/1 - x, according to equation (3),

$$x/(1-x) = \left(1 - \frac{v_p}{v}\right) / \left(\frac{v_1}{v} - 1\right) \tag{4}$$

³ Sörensen, S. P. L., and Sörensen, M. H., Compt. rend. Lab. Carlsberg, 15, No. 9 (1924).

⁶ Sörensen, S. P. L., and Sörensen, M. H., Compt. rend. Lab. Carlsberg, 19, No. 11 (1933).

Replacing the specific volumes by the densities we have

$$w = \left(\frac{D_p - D}{D - D_1}\right) \frac{D_1}{D_p} \tag{5}$$

where D = density of the crystal, $D_p = \text{density}$ of anhydrous protein $= 1/v_p$, and $D_1 = \text{density}$ of water. Values for various proteins which have been calculated with the aid of equation (5) are of the same order of magnitude as those which are given in Table V.

The fact that the densities of protein crystals are less than the apparent densities of the dry solid proteins may be explained on the basis that protein crystals contain water of hydration. However, the sense in which the latter term is used may not be the same as that which it implies when we speak of water of hydration of a salt such as copper sulfate. The water of hydration of proteins is apparently not dependent upon an orderly arrangement of crystal forces since heat coagulated proteins contain the greater part of the water which is present in the crystal form. It is possible, in accordance with the lattice-space concept of protein structures, that water is free to diffuse in and out of the spaces, but the spaces themselves are always filled with fluid. An alternative hypothesis is that the protein contracts when it is dissolved. However, as seen from the data which are given in Table VI, there is no experimental

Table VI

The Partial Specific Volume of Dry Protein in Suspension and in Solution

Protein	Horse hemoglobin	Edestin
Temperature (degrees)	1.0	21.9
of hemoglobin	1.344	1.349
pension	0.744	0.741
Partial specific volume of dry protein in solution	0.742	0.745
Contraction in ml./gm. crystalline protein dissolved	0.002	-0.003

(Adair, G. S., and Adair, M. E., Proc. Roy. Soc. London, 120 B, 422 (1936).)

evidence to support this view. The data are not, as yet, sufficiently extensive to draw any conclusions as to the possibility that proteins are hydrated in solution.

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SECTION IX. MAGNETIC AND DIAMAGNETIC PROPERTIES

1. PROPERTIES OF HEMOGLOBIN

Although hemoglobin contains iron, it is nevertheless decidedly diamagnetic. This statement is based on the extended series of studies by Gamgee (1) who came to the conclusion that methemoglobin and carbon monoxide hemoglobin as well as oxyhemoglobin are strongly diamagnetic compounds. On the other hand,

the iron-containing radical, hematin (or heme), is strongly magnetic. According to Benedicenti (2) (3), the addition of protein inhibits the lowering of the diamagnetic constant of water which results on the addition of ferric chloride or finely divided iron. Presumably this is due to the formation of an iron-containing protein compound (see Chapter XIII).

Pauling and his co-workers (4) have recently reported studies on the magnetic properties of several of the hemoglobins. They find that the carbon monoxide hemoglobin molecule has zero magnetic moment and hence contains no unpaired electrons. This is interpreted as showing that at least two 3 d orbitals of each ferrous iron atom are involved in covalent bond formation, the atom presumably forming six octahedral d^2sp^3 bonds, four to the porphyrin nitrogen atoms, one probably to a nitrogen atom of the globin, and one to the carbon monoxide molecule. They assign the resonating structure to carbon monoxide hemoglobin indicated by the formulas:

in which the dashes represent shared electron pairs and the dots unshared electrons.

Oxyhemoglobin also has zero magnetic moment and contains no unpaired electrons. Each iron atom is attached to the four porphyrin nitrogen atoms, the globin molecule, and the oxygen molecule by covalent bonds. The structure of oxyhemoglobin is probably analogous to that of carbon monoxide hemoglobin and it is likewise resonating. The structure may be represented by the formulas:

Reduced hemoglobin contains unpaired electrons. Its magnetic susceptibility corresponds to an effective magnetic moment of 5.46

Bohr magnetons per heme, calculated for independent hemes, thus showing the presence of four unpaired electrons per heme, and indicating that the heme-heme interaction tends to stabilize to some extent the parallel configuration of the moments of the four hemes in the molecule.

In hemoglobin the bonds from iron to the surrounding atoms are ionic, while in oxyhemoglobin and carbon monoxide hemoglobin they are covalent.

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SECTION X. THIXOTROPY

1. PROPERTIES SHOWN BY AMINO ACIDS AND PROTEINS

If an inorganic electrolyte, such as sodium chloride, is added to a concentrated ferric oxide sol in an amount which is insufficient to produce coagulation, a gel which has a paste-like consistency results. If this gel is shaken, a fluid which has all of the properties of the original sol is formed. On standing, a gel again forms. This process may be repeated. The time which is required for the formation of a gel is fairly constant and, up to a certain limit of concentration of electrolyte, decreases with increasing concentration of electrolyte.

According to Freundlich and Rosenthal (1) the presence of amino acids increases the time which is required for gel formation, i.e., retards gelation. This effect is explained as being due to the formation of iron-containing complexes on the surface of the colloid particles. Increase in the concentration of hydrogen ions increases the time of setting of the sol (2).

The phenomenon of thixotropy is quite a general one. It has been particularly observed in the case of aluminum, iron, and copper hydroxide sols (3).

Freundlich and Abramson (4) have shown that gelatin gels are thixotropic. However, the gel formation takes place so rapidly that, under ordinary experimental conditions, the phenomenon is not easily discernible. Thixotropy can be produced by cataphoresis. If a gelatin gel of suitable concentration containing suspended particles, such as powdered quartz, is frozen and then subjected to cataphoresis, the velocity of the particles decreases to a smaller constant value. Passage through a capillary produces thixotropic behavior in solutions of gelatin and dibenzoyleystine.

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SECTION XI. MELTING AND DECOMPOSITION POINTS OF AMINO ACIDS

1. GENERAL CONSIDERATIONS

The melting point of none of the amino acids is sharp; most of them decompose at or near the melting point. It is obvious that the reported melting points of the amino acids which have been determined by the usual methods are not reliable. Moreover, many of the data that are found in the literature were probably obtained on products which were not in the highest state of purity or free from traces of moisture; other data were not corrected for barometric pressure or exposure of the thermometer. In some instances it was not stated whether the melting point estimation was carried out in an open or in a closed tube; whether the material was finely ground; or whether the heating was carried out slowly or rapidly. The various factors indicated above no doubt account for the great variability in the melting point data which is to be found in the literature. That certain of these factors came into play is seen from the fact that, in a number of instances, different melting points have been reported for the d- and l-isomers of an amino acid. There is no reason to expect a difference. In the case of racemic amino acids the melting point may be influenced by the fact that a racemic compound may be formed between the two isomers.

Dunn and Brophy (1) have attempted to circumvent certain of the difficulties in determining the melting points of amino acids by producing the change in the physical state of the amino acid almost instantly. This is essentially the basis of the procedure used by Dennis and Shelton (2). Dunn and Brophy determine with a high degree of precision the time and temperature required for the amino acid to reach a standard decomposition state as judged by

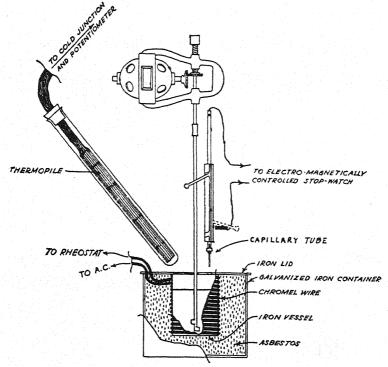


Fig. 1. Apparatus used for determining melting points. (Dunn, M. S., and Brophy, T. W., J. Biol. Chem., 99, 221 (1933).)

the shade of yellow or brown color produced. The time-temperature values are plotted and the decomposition point of the amino acid is taken to be that temperature at which the curve becomes parallel to the base axis. It is claimed that a standard color can be reproduced to ± 0.1 second and $\pm 0.1^{\circ}$. The apparatus used by Dunn and Brophy is illustrated in Fig. 1.

A compilation of melting and decomposition point data is given in Table I. No attempt has been made to give all of the values which are to be found in the literature. The data are presented in order to point out the need of establishing, by means of a method which will yield reproducible data of a high degree of accuracy, the change in the physical state of amino acids which is designated as the temperature at which the compound melts or rather begins to decompose.

Table I

Melting and Decomposition Points of Amino Acids and Related Compounds¹

Compound	Melting or Decomposition Point degrees	Compound	Melting or Decomposition Point degrees
d-Alanine	297 d	<i>l</i> -Isoleucine	285-286 d
l-Alanine	295 d	l-Isoleucine (allo)	278 d
d,l-Alanine	295, 297 D	d-Isoleucine (allo)	274-275 d
d - α -Amino- n -butyric acid	303 d	d,l-Leucine	332 D 290
$d, l-\alpha$ -Amino- n -butyric acid.	299	l-Leucine	293–295 d
d, l - α -Aminoisobutyric acid.	319-320		337 D
d, l - β -Amino- n -butyric acid.	191-192	d-Lysine	224 d
$d, l-\gamma$ -Amino-n-butyric acid.	203	<i>l</i> -Lysine	224 d
α -Amino- γ -hydroxybutyric acid		<i>l</i> -Lysine picrate	240 darkens 265-266 ex-
d,l-Arginine	and the second of the second of		plodes
d-Arginine		d,l-Methionine	281
d,l-Aspartic acid		l-Methionine	275-277
d-Aspartic acid		d,l-Phenylalanine	318-320 D
l-Aspartic acid	269-271	스템 보스템이 보고.	260-263
l-Asparagine		d-Phenylalanine	283-284 d
l-Canaline		l-Phenylalanine	283 d
d,l-Canavanine flavianate	215	d,l-Proline	205 d
d,l-Cystine		d-Proline	215-220 d
d-Cystine	247-249	l-Proline	220-222 d
<i>l</i> -Cystine		d,l-Norleucine	327 D
			294-296
Citrulline	202	d-Norleucine	301 d
l-Dihydroxyphenylalanine.	280 d	l-Norleucine	301 d

¹ d = decomposition; D = decomposition point from Dunn, M. S., and Brophy, T. W., J. Biol. Chem., 99, 221 (1933).

Although, in some cases, no statement is given that decomposition takes place at the given melting point, it is very probable that it does so. Due to the fact that the melting point is usually not very sharp, that decomposition may set in before the compound melts and decomposes, and that, in some instances, the products were not pure, most of the melting point data cannot be considered as being very precise.

The list of references which give melting points of amino acids is too extensive to be included here. Access to the literature may be gained by consulting the list of references which are given by Vickery, H. B., and Schmidt, C. L. A., Chem. Rev., 9, 169 (1931).

Table I—Continued

Melting and Decomposition Points of Amino Acids and
Related Compounds—(Continued)

Compound	Melting or Decomposition Point degrees	Compoun d	Melting or Decomposition Point degrees
<i>l</i> -Diiodotyrosine	239–241 D 202 d	d,l-Serine	246 d
d,l-Glutamic acid	225–227 D 198	d-Serine	228 d
d-Glutamic acid	247-249 D	l-Serine	223 d
	206	d,l-Tryptophane	283-285
l-Glutamic acid	213 d	d-Tryptophane	281-282
Glycine	233 d	l-Tryptophane	281-282
시계에 이 얼마나 생각되었다.	289-292 D		289 d
d,l-Histidine	285-286 d	l-Threonine	255-257 d
d-Histidine		l-Thyroxine	235-236 d
l-Histidine	265 d	d-Thyroxine	237
l-Homocystine	232-233	d,l-Tyrosine	316
d,l-β-Hydroxyglutamic acid	loses H ₂ O, 100 198 d	d-Tyrosine	310-314
d - β -Hydroxyglutamic acid.	loses H ₂ O, 100	l-Tyrosine	295 d
			342-344 D
l-Hydroxyproline	270 d	d,l-Valine	298 d
	238-241 d		292 D
d-Hydroxyproline	274 238–241 d	d-Valine	315 d
d,l-Isoleucine	292 D	l-Valine	293 d
d-Isoleucine	283-284 d	Glycylglycine	262-264 D 236
		Glycylglycylglycine	7.7

In the case of the amino acids this property should not, at present, be used as a criterion for the purity of a particular product. The decomposition points of a number of the amino acids do not lie sufficiently far apart so that the decomposition point will be materially influenced by admixture of small amounts of another amino acid having a decomposition point not far removed from that of the one to be tested. Dunn and Brophy have shown that the decomposition point of glycine was only lowered from (289° to 292°) to (284° to 286°) when 1.4 per cent of ammonium chloride was present, and to (247° to 249°) when 10.7 per cent of this substance was present. It is quite necessary that other criteria be used in addition to the temperature of decomposition in order to establish the purity and identity of an amino acid.

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SECTION XII. ANISOTROPY

By JOHN T. EDSALL

(Department of Physical Chemistry, Harvard Medical School, Boston)

1. ANISOTROPY OF TISSUES

All gases, most liquids, and some solids are, under ordinary circumstances, isotropic; that is, their reaction to a physical disturbance (such as a mechanical stress or a beam of light) is independent of the direction from which the disturbance proceeds. The great majority of crystals, however, are anisotropic. Thus the resistance of a crystal to mechanical stress depends on the direction in which the stress is applied, and is in general different along the different axes of the crystal; the induced electric polarization in an electric field will be different in different directions; and the velocity with which a beam of light passes through the crystal will depend on its direction of incidence. All these and other phenomena are manifestations of anisotropy; we may thus speak of mechanical anisotropy, electrical anisotropy, optical anisotropy, and so forth. All of these, however, stand in the most intimate relation to one another; all depend upon the existence of a definite type of pattern and organization in anisotropic systems, which marks them out as having characteristic directions, or axes, in terms of which their organization may be described.

Organization and pattern are pre-eminently characteristic of living tissues; it is therefore to be expected that many such tissues should show the phenomenon of anisotropy (1). It is indeed clear even to casual observation that a muscle fibre or tendon is mechanically anisotropic; the response of a muscle to a stress applied parallel to the axis of the fibre will be quite different from its response if the stress is at right angles to the axis. Likewise, smooth muscle and the "dark" bands of striated muscle are optically aniso-

¹ Substances initially isotropic may become anisotropic under mechanical stress (as in the well-known "photoelastic effect" in glass) or in an electric field (the Kerr electric effect).

tropic: if light passes through in a direction perpendicular to the long axis of the fibre, the index of refraction (which is inversely proportional to the velocity with which light travels through the substance) is greater for plane-polarized light which vibrates parallel to the long axis than for light which vibrates at right angles to it.² This difference of refractive index in different directions is the optical manifestation of anisotropy; it is known as double refraction. The presence of double refraction in muscle was discovered by Brücke (2) in 1858.

In a doubly refractive system, there is one direction, known as the optic axis,³ along which light is always propagated with the same speed, regardless of its state of polarization. In the muscle fibre, this optic axis coincides with the mechanical axis of the fibre. The magnitude of double refraction is measured by the difference between the index of refraction (n_e) for light whose electric vector vibrates parallel to the optic axis, and the index (n_0) for light which vibrates perpendicular to it. If $n_e > n_o$, the double refraction is positive; if $n_e < n_o$, negative. The double refraction of muscle is positive; $n_e - n_o = 0.0025 - 0.0045$. Lipids often form systems showing negative double refraction.⁴

The double refraction of a tissue fibre may be measured as follows. The fibre is laid on the stage of a microscope, with its long axis parallel to the plane of the stage. The light passing up through it from below is made plane polarized by a Nicol prism or other polarizing device (the polarizer); between the object studied and the eyepiece is inserted another Nicol prism (the analyzer) which is set so as to extinguish the light coming from the polarizer. The fibre is adjusted so that the plane of polarization of the light passing through the polarizer is at 45° to the axis of the fibre. On entering the tissue, the light is now resolved into two components of equal intensity; one vibrating parallel to the axis and travelling with velocity proportional to $1/n_e$, and one perpendicular to it, travelling with velocity proportional to $1/n_e$. Since one component

² In speaking of the direction of vibration, we are referring to the electric vector of the light wave. This vector is, of course, always at right angles to the direction of propagation of the light.

³ Many crystals, known as biaxial, possess two optic axes and require three different refractive indices for their characterization. For the purposes of this discussion, however, we need consider only uniaxial systems, which may be characterized in terms of one optic axis and two refractive indices.

^{*} For a more thorough discussion of double refraction, see any standard treatise on optics; for instance, Wood, R. W., Physical Optics, 3rd ed., New York, 1934.

travels faster than the other, the two will be out of phase when they emerge from the tissue; they will compound into a resultant vibration which is in general no longer plane polarized, but elliptically polarized. As a result, the doubly refractive tissue under the microscope appears bright on a dark background. The same superficial appearance might be produced by optical rotation, but the two phenomena can readily be distinguished. If the tissue had simply rotated the plane of polarization of the incident light, rotation of the analyzer through a suitable angle would again make the tissue appear dark. This, however, is not the case; the tissue still appears bright for every possible position of the analyzer. The light can be extinguished, however, by placing a suitable crystal (or other doubly refractive material) between tissue and analyzer, and adjusting it so as to produce an elliptical polarization equal and opposite to that arising from the substance under study. Such a device is known as a compensator, and when suitably calibrated, it can be used for the quantitative measurement of double refraction. Indeed, if the thickness of the tissue studied is known, and the phase difference in the elliptically polarized light it produces is determined, the double refraction of the tissue can be calculated immediately (3).

Double refraction is a characteristic property of all types of contractile tissue, as the work of Engelmann (4) on a very wide variety of tissues has shown. Striated muscle is unusual among such tissues in that it possesses isotropic elements (the I discs) alternating with the anisotropic (Q) bands.

Thus there is good reason for believing that an intimate connection exists between double refraction and the processes directly involved in muscular contraction. The sudden decrease in double refraction associated with the onset of the contractile process, and its subsequent return to normal after the contraction have long been known. They have been carefully studied by von Muralt (5) and show a striking correlation with other physical features underlying contraction. Especially striking is the fact that the change in double refraction on stimulation persists even when the mechanical response is paralyzed by cane sugar or other agents. These phenomena prompt an inquiry as to the fundamental nature of the physical system underlying the phenomenon of double refraction in muscle.

Brücke (2) suggested that the double refraction of muscle is due to a submicroscopic pattern of small crystalline elements,

arranged with their axes parallel to one another. In spite of the plausibility of this hypothesis, however, no decisive evidence in its favor was forthcoming until 1923. In this year, Stübel (6) published studies on the double refraction of muscle fibres fixed by histological techniques and then imbedded in a series of solvents of

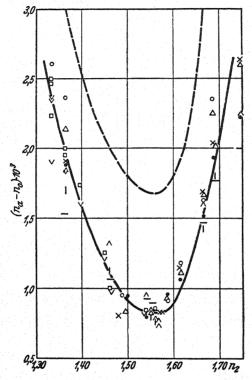


Fig. 1. The total double refraction of the resting muscle fibre. Abscissa: N₂, refractive index of imbibition fluid. Ordinate: double refraction. Lower curve shows results of measurements on nine different muscle fibres. Upper dotted curve: myosin fibre, for comparison.

(Noll, D., and Weber, H. H., Arch. ges. Physiol., 235, 234 (1934-35).)

differing refractive index. In making his observations, he was guided by a theory proposed by Wiener (7) who showed that a system composed of parallel isotropic rod-shaped elements (refractive index n_1), small compared with the wave-length of light, and imbedded in a continuous medium of refractive index n_2 , should be doubly refractive if $n_1 \neq n_2$. This is true even though all of the elements of the system be inherently isotropic; the double refraction being given by the formula:

$$n_e^2 - n_0^2 = \frac{V_1 V_2 (n_1^2 - n_2^2)^2}{(V_1 + 1)n_2^2 + V_2 n_1^2} \tag{1}$$

where V_1 is the volume fraction of the rod-shaped elements, and V_2 that of the surrounding medium. This "structural double refraction" ("Stäbchendoppelbrechung.") is zero when $n_2 = n_1$, and rises in an approximately parabolic curve for lower or higher values of n_2 . If the rods themselves are inherently anisotropic, the curve will pass through a finite minimum value when $n_2 = n_1$ instead of descending to zero. Stübel showed clearly that the double refraction of muscle fibre does indeed vary with the refractive index of the fluid in which it is imbedded, much as would be expected from equation (1). The type of curve obtained is shown in Fig. 1, taken from the more recent and accurate work of Noll and Weber (8).

2. ANISOTROPY OF MYOSIN

Stübel's experiments, in conjunction with Wiener's theory, supplied a solid foundation for the belief that long rod-shaped molecules, oriented in parallel, were responsible for double refraction in the muscle fibre. The chemical nature of the molecules in question was, however, still unknown; and the first strong evidence concerning their identity was furnished several years later when it was shown that myosin, the globulin of muscle, exhibits the phenomenon known as double refraction of flow (9). Myosin is a typical globulin, insoluble in distilled water, soluble in salt solutions of moderate concentration; it is extremely viscous, and in the absence of salt readily forms gels (10). In solution, myosin appears completely isotropic when at rest, but if the solution is stirred with a glass rod, it immediately shows well-marked double refraction, which disappears gradually over the course of several seconds when the stirring ceases.

This phenomenon was already known for a number of colloids, being particularly marked in sols of vanadium pentoxide, which have been intensively studied by Freundlich, Stapelfeld and Zocher (11) and Freundlich, Neukircher and Zocher (12). These sols are known to consist of long thin asymmetric particles, and the double refraction produced by flow was attributed to orientation of the colloidal particles produced by the shearing stresses arising during flow. At rest, the individual particles are anisotropic, but they are oriented at random and their individual effects neutralize one another. The same explanation of double refraction of flow in

myosin was maintained by von Muralt and Edsall (9). The diagram (Fig. 2) taken from their work, illustrates the underlying conception. The myosin solution is placed in the space between two concentric cylinders, and observed between crossed Nicol prisms placed above and below. When the liquid is at rest, the whole field of view is dark; but, on setting the outer cylinder into rotation, the field lights up, except for a dark cross with four arms (Fig. 2). This "cross of isocline" represents the points at which the optic axis of the oriented myosin solution coincides with the plane of

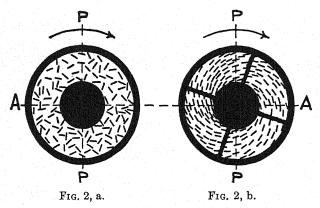


Fig. 2. Orientation of particles in a doubly refractive fluid placed between concentric cylinders; Fig. 2, a, particles, each schematically represented by a line indicating its optic axis, at rest; Fig. 2, b, orientation due to motion of external cylinder.

(Muralt, A. L. von, and Edsall, J. T., J. Biol. Chem., 89, 315 (1930).)

polarization of polarizer (PP) and analyzer (AA). The angle of isocline, Ψ , is defined as the larger of the two angles which the "cross of isocline" forms with the planes AA and PP. The two variables requisite to describe the anisotropy of a solution showing double refraction of flow are the angle of isocline (Ψ) and the magnitude (Δ) of the double refraction. These are functions of the velocity gradient in the flowing liquid (determined at any point by the speed of rotation of the outer cylinder), of the viscosity of the liquid, of the concentration of the doubly refractive colloid, and of the temperature. In many instances (as in the V_2O_5 sol), they depend also on the history or "age" of the colloidal solution, the size of the particles changing with time. A given myosin solution, however, shows behavior which is virtually independent of "age," provided care be taken to avoid denaturation.

In dilute solutions of myosin at low velocity gradients, the

angle Ψ approaches a limiting value of 45° (Fig. 3). As the concentration of the solution and the velocity gradient increase, Ψ increases but apparently never surpasses a limiting value of approximately 78°. (Egg globulin solutions (13) attain the maximum possible value for Ψ , namely 90°). Increase in the viscosity of the solution tends to increase Ψ ; rise of temperature to decrease it.

The value of the double refraction of flow in myosin (Δ) as a function of concentration and velocity gradient is shown in Fig. 4. As the velocity gradient increases from zero, Δ rises rapidly at first and then more slowly. It is not certain whether the curves are

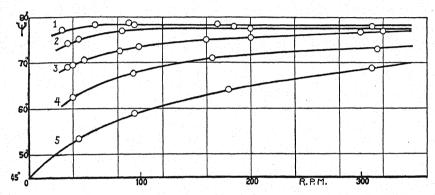


Fig. 3. The angle of isocline Ψ is plotted against the speed of rotation of the external cylinder for various concentrations of a myosin preparation from rabbit muscle. Curve 1, concentration 2,240 mg. of N per liter, $\eta'_{\rm rel.}$ 16.5; Curve 2, concentration 1790 mg. of N per liter, $\eta'_{\rm rel.}$ 10.5; Curve 3, concentration 1,345 mg. of N per liter, $\eta'_{\rm rel.}$ 6.5; Curve 4, concentration 450 mg. of N per liter, $\eta'_{\rm rel.}$ 2.2; Curve 5, concentration 225 mg. of N per liter, $\eta'_{\rm rel.}$ 1.48.

(Muralt, A. L. von, and Edsall, J. T., J. Biol. Chem., 89, 315 (1930).)

tending toward an upper limiting value at high velocity gradients (a value which would presumably correspond to complete orientation of the anisotropic particles), or whether the upward course of the curve would continue indefinitely. Increase of viscosity of the solution and decrease of temperature tend to increase the value of Δ .

Denaturation of myosin is accompanied by a loss of its power to produce double refraction of flow. Such typical denaturing agents as concentrated urea solutions, acids and alkalies, iodides, and thiocyanates all produce rapid and complete disappearance of the double refraction. Weber and Stöver (14) have found that concentrated urea solutions reduce the molecular weight of myosin (determined by osmotic pressure) from about 1,000,000 to about

100,000. Thus far, this denaturation phenomenon has not been shown to be reversible.

3. THEORY OF DOUBLE REFRACTION OF FLOW

Since the experimental studies on myosin, quantitative theories have been developed, relating the observed angle of isocline and double refraction to the magnitude of the velocity gradient and to

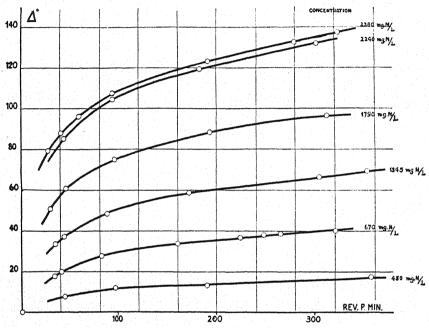


Fig. 4. Double refraction of rabbit myosin solution, plotted against speed of rotation of outer cylinder, for various myosin concentrations.

(Muralt, A. L. von, and Edsall, J. T., J. Biol. Chem., 89, 351 (1930).)

the Brownian movement of the colloidal particles, which depends upon their size and shape (15, 16, 17). Double refraction in flowing liquids may be due either to orientation of inherently anisotropic particles, or to distortion of initially isotropic particles, by the shearing stresses arising in the flowing solution. The latter effect might occur in a protein composed of a long polypeptide chain, which had become curled and twisted by rotation around valence bonds into a nearly spherical (isotropic) shape. Application of a suitable shearing stress might cause the molecule to uncurl and become markedly anisotropic.

⁵ Similarly an electric field acting on a group of molecules produces polarization due both to orientation of permanent dipoles, and to the production of induced dipoles by distortion. (Debye, P.)

Qualitatively, the phenomena observed in flowing solutions of myosin can be explained on either theory. The form of the experimental curves (Figs. 3 and 4) does indeed correspond quite closely with the curves deduced by Boeder (15) from the orientation theory. On the other hand, Haller (17) believes the behavior of myosin solutions to be explicable in terms of the deformation of isotropic particles. The extreme ease with which double refraction can be produced, however, by very small shearing stresses in a dilute (<1 per cent) solution of myosin, seems very difficult to interpret except in terms of orientation of anisotropic particles. The conclusion of von Muralt and Edsall, that the double refraction of myosin is due primarily to an orientation effect, on which a smaller distortion effect is superposed, still appears to be compatible with all the available evidence.

On this basis, the theory of Kuhn (16) permits a rough estimate of the length of the myosin molecule, which is calculated from the experimental data as being roughly 6,000 Å. This is about half the length of the anisotropic (Q) band in frog striated muscle, which had been estimated by Hürthle (18) as about 10,000 -11,000 Å $(1.0-1.1\mu)$.

4. MYOSIN FIBRES

An important advance was made by Weber (19), who produced anisotropic myosin fibres possessing many of the properties of muscle fibres. They are very simply prepared: a solution of myosin, dissolved in salt, is squirted through a fine capillary into distilled water, in which, being insoluble, it coagulates in the form of a fine thread. These threads have appreciable tensile strength, being capable of supporting their own weight up to a length of a metre, even though containing only about 1 per cent protein; on being dried under slight tension, they show strong positive double refraction and high tensile strength. Slight moistening and further stretching of the fibres appears to bring about nearly complete orientation of the protein; such fibres give an X-ray diffraction diagram virtually identical with those of muscle (20). Even more important is the optical behavior of these fibres. Immersed in fluids of varying refractive index n₂ (time being given for the immersion fluid to penetrate completely into the interior of the fibre), they give an n_2 -double refraction curve (Fig. 5) very similar to that of the intact muscle fibre (cf. Fig. 1). (Compare discussion of Stübel's work, above.) In both cases, the minimum of the curve

comes at $n_2 = 1.576$; in both cases, the double refraction at the minimum is quite large, indicating that in addition to the "structural double refraction" due to the parallel arrangement of the myosin molecules in the fibre, there is an intrinsic double refraction ("Eigendoppelbrechung") due to the anisotropic nature of the myosin molecules themselves. The absolute value of the double refraction in the myosin fibre is higher than in intact

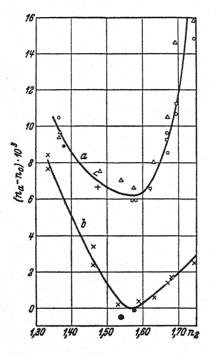


Fig. 5. Double refraction of myosin fibre (a) and gelatin fibre (b), as a function of refractive index (n₂) of imbibition fluid. Abscissa and ordinate same as in Fig. 1. (Weber, H. H., Arch. ges. Physiol., 235, 205 (1934-35).)

muscle, because of its high myosin and low water content; the ratio of double refraction to myosin content is not far from equal in the two systems. Weber has also studied the elastic properties of myosin fibres, and the heat contracture which they undergo, in which they bear a suggestive resemblance to the intact muscle fibre. Meyer (21) has indeed suggested that the contraction of muscle is directly produced by a sudden shortening of the long anisotropic protein molecules in the fibre; and, although his theory is open to objection in detail, it remains the most suggestive hypothesis concerning muscular contraction that has yet been advanced.

The distinctive anisotropy of the myosin fibre is shown by comparison with a similar fibre made from gelatin. (Fig. 5). Not only is the n_2 -double refraction curve for gelatin of a quite different form, but the double refraction descends to zero at the minimum of the curve, indicating that the gelatin molecules (or micelles) of which the fibre is composed are intrinsically isotropic (22).

5. OTHER ANISOTROPIC PROTEINS

It has been shown by Boehm and Signer (23) that egg globulin and fibrinogen show double refraction of flow, although in both cases the magnitude of the effect is much less than for myosin. These proteins also may therefore be considered anisotropic. Boehm and Signer believe that the egg globulin molecule is even longer and more asymmetrical than that of myosin, because it shows an angle of isocline of 90°; but, in view of its markedly smaller double refraction, this conclusion cannot vet be considered as established. Egg albumin, casein, serum albumin and globulin, hemoglobin and the muscle proteins, myogen and globulin X, show no trace of double refraction of flow, and are therefore not highly asymmetrical. Svedberg (24) has indeed concluded that the egg albumin molecule is spherical, from its behavior in diffusion experiments and in the ultracentrifuge. On the other hand, casein and hemoglobin appear from the ultracentrifugal studies to be definitely asymmetrical. Since neither of these proteins shows double refraction of flow, we must conclude that a strongly doubly refractive protein like myosin must be very asymmetrical indeed.

A number of the long-chain polymers—polystyrol, polyvinyl alcohol and others—synthesized by Staudinger and his collaborators, have been shown (25) to give double refraction of flow, which in its general character is closely akin to that of the anisotropic proteins.

The anisotropy of many tissues indicates the possibility of extracting and studying many anisotropic proteins not yet known. The investigations of Schmitt and his collaborators (26) on the anisotropy of nerve promise to yield results of great interest. The weakly positive double refraction of the axis cylinder appears to be due to protein; while the negative double refraction of the medullary sheath in which the birefringent elements are radially oriented is apparently due to a negative lipid and a positive protein component, the former predominating. Preliminary studies have been made of some of the anisotropic proteins involved. It

may well be expected that similar proteins will be found as structural elements in many other tissues.^{6,7}

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SECTIONS XIII TO XV (INCL.)

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SECTION XIII. THE HOFMEISTER IONIC SERIES

1. EFFECTS OF SPECIFIC IONS UPON PROTEINS

In 1887 there appeared the first (1) of a series (2) of articles from Hofmeister's laboratory dealing with the effects of specific ions upon certain of the physical properties of proteins, particularly the precipitation of a number of proteins such as serum globulin, ovalbumin, and gelatin, and the swelling of gelatin gels. The results have, in a general way, been confirmed by others (3). Similar ionic effects have been reported for the osmotic pressure and viscosity (4) of protein solutions.

The observations may be summarized briefly by the statement that when salts of the same cation are used, the anions can be arranged in a definite series according to the relative influence which they have on the viscosity, swelling, precipitation, and certain other properties of proteins. The arrangement of the anions for the salting-out and depression of the osmotic pressure of proteins is approximately: SO_4 >citrate>tartarate>acetate> $Cl>NO_3>Br>I>CNS$, where the sulfate ion shows the greatest and the thiocyanate ion the least effect. The influence of the ions on the swelling of gelatin is approximately in the reverse order to that given above. The effect of salts upon the viscosity of protein solutions is discussed in Section V of this chapter.

The differences between the effects produced by different cations, when combined with the same anion, are less marked than in the case of the anionic series. The order may be expressed approximately as $K > Rb > Na > Cs > Li > NH_4$. The specific ionic effects have come to be known as the Hofmeister series of ions or the lyotropic series.

Various theories have been proposed to explain the effects produced by the Hofmeister ionic series. These may be briefly summarized as follows: (a) the effects of the ions are in the order of their degree of hydration (5). While hydration of ions does occur, the amount of hydration is not strictly proportional to the specific influences exerted by the ions according to the Hofmeister series (6); (b) in addition to hydration of the ions, orientation of the salt molecules in the surface film and orientation of the water molecules surrounding the ions of the salt have been considered as factors which influence the phenomenon (7); (c) the effect is due to the intensity of absorption of the ions concerned; (d) the effects of neutral salts in precipitating proteins depend upon the size of the ions and their influence upon the dielectric constant of the protein solutions (9).

Loeb (10) denied the existence of the Hofmeister series particularly with respect to the influence of the ions on osmotic pressure, viscosity, and swelling of proteins. He attributes the observed effects as being due to a neglect on the part of experimenters to take into consideration the influence of pH and the valence of the ions. He states, "In the development of these Hofmeister series, a serious error has been committed, namely, the neglect of measuring the hydrogen ion concentration of the protein solutions and protein gels and of comparing the effect of ions at the same pH of the protein solution or protein gel. As a consequence, effects which are due only to variations in the hydrogen ion concentration were erroneously ascribed to the chemical nature of the anion. If the hydrogen ion concentrations are duly measured with the hydrogen electrode and properly taken into consideration, it is found that certain properties of proteins are influenced only by the valency

of the ion and not by its chemical nature; and that, further, in these cases only that ion has any effect the sign of charge of which is opposite to that of the protein ion."

Loeb's conclusions were supported by experiments which showed that, at the same pH, cations of the same valency depress the osmotic pressure of gelatin chloride to the same extent. Similarly, the depression of the viscosity and the swelling of gelatin chloride, when the pH was kept constant, were, within the limits of his experimental error, determined by the valency of the ions and not by their chemical nature. However, in the case of swelling of gelatin the effect of acetic acid was slightly greater than that of the other monobasic acids. He explained this as being due to the high concentration of acetic acid which was necessary to bring the pH of the gel to pH 3.2 or 3.0 and which diminished the cohesion of the gel. Above pH 8.5 the influence of ammonia on the swelling of gelatin was irregular. This apparently was due to the high concentration of this base which was necessary to bring the gelatin to this pH. On the basis of Loeb's experiments it is evident that pH and valency of the ions must be taken into consideration in dealing with certain of the effects of the ions in the Hofmeister series.

Gortner, Hoffman, and Sinclair (7) determined the amount of protein which was extracted from a number of samples of wheat flour by various salts. The pH was carefully controlled. They found that the anions arrange themselves, in the order of increasing peptizing effect, as follows: $F < SO_4 < Cl < tartarate < Br < I$. Although the effect, in the case of the cations, was less marked than in the case of the anions, the order of increasing peptizing action was Na < K < Li < Ba < Sr < Mg < Ca. Variation in pH was not considered to be sufficient to account for the observed differences in the ionic efficiency.

The data of Green (11) and McMeekin (12), which are represented graphically in Figs. 16a and 16b of Chapter XVI, show quite definitely that, at the same ionic strengths, the solvent action of certain salts on cystine and hemoglobin decreases in the order NaCl > (NH₄)₂SO₄ > Na₂SO₄. The magnitude of the effects is far greater in the case of hemoglobin than in the case of cystine. A more complete discussion of the influence of salts upon the solubility and salting-out of proteins is given in Section III of Chapter XVI.

The effects of the Hofmeister ionic series are too striking to be ignored, to be dismissed as being fictitious, or attributed solely to

pH and the valency of the ions. It is true that they become conspicuous only at a considerable concentration of electrolyte and tend to disappear in dilute solution. It is possible that too many of the properties of proteins have been attributed to specific ionic effects. Undoubtedly, more studies with respect to each of the properties of proteins, upon which specific ionic effects have been reported, will have to be carried out before the phenomenon is fully understood. Concentration of the protein as well as its chemical nature should be taken into consideration if quantitative as well as qualitative differences in the specific effects of ions are to be determined.

From the present state of our knowledge it is quite apparent that the various phenomena upon which the Hofmeister ionic series have been considered to have an influence are quite complex. This can be said particularly of the solubility and salting-out of proteins. "Dehydration" of the protein by the added salt in the sense that the ions attract the more polarizable water molecules about themselves probably plays an important rôle (see Chapter XVI, Section III). However, until more adequate data are available, the explanation of the effects produced by the Hofmeister series of ions must be left in abeyance.

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SECTION XIV. ANTAGONISM OF IONS

1. CHEMICAL ASPECTS

A notable contribution to biology was made by Ringer (1) when he observed, on perfusing a frog's heart with physiological salt solution, that, although the beat might continue for some time, it became feebler with time and finally ceased altogether. If small amounts of calcium or potassium chloride were added to the salt solution the heart beat was maintained much better. A somewhat similar interrelationship between salts was observed by Loeb (2) when he noted that a small marine fish, Fundulus, when placed in a solution of sodium chloride which was isotonic with sea water, soon died; but the fish lived indefinitely when potassium and calcium chloride were added in the right proportions. This phenomenon has been termed salt or ion antagonism.

Simms (3) has carried out a series of experiments in which he attempted to throw some light on the nature of ion antagonism. He first showed (4) that the monoions of monovalent and polyvalent weak acids in the presence of Na+ or Mg++ obey the limiting Debye-Hückel equation when the acids are in dilute aqueous solution. On the other hand, diions and triions of weak acids in the presence of Na+ obey a modified equation in which a correction for the distance between the like charges is introduced. The presence of Mg++ causes a marked decrease in the activity of the di- or triion which is not explained by the Debye-Hückel theory. The effect is very great with short-chain acids, particularly oxalic acid. The addition of sodium or potassium ions caused a marked diminution in the effect of Mg++ on the activity of the oxalate diions. This fact can be expressed mathematically by the equation,

$$k = \frac{\text{Inactivated Ox}^{-}}{\text{Active Ox}^{-}} \frac{\text{Na}^{+} + \text{K}^{+} + 2\text{Mg}^{++} + A}{\gamma_{\text{Mg}} (\text{Ox}^{-})^{1.5}}$$
(1)

where A is a constant which changes with the concentration of oxalate ions. This equation was shown to hold down to zero ionic strength of Na⁺ and K⁺.

Sulfate ions caused an increase in the pK_1' and pK_2' values of malonic acid above those required by the Debye-Hückel equation. In the case of oxalic acid the effect of SO_4 on the pK_2' value of oxalic acid increased with the concentration of SO_4 according to the equation,

$$k = (f'-1)\frac{2}{(SO_4^{-})^{1/2}}$$
 (2)

where f' is the antilog of the increase in pK₂' due to the presence of SO₄=. The presence of NaCl antagonized the effect of Na₂SO₄ according to the equation,

$$k = (f'-1) \frac{2SO_4 = +Cl - +A}{(SO_4 =)^{1.5}}$$
(3)

In other words, sulfate ions produced an anomalous effect on the ionization of the oxalate diions which was opposite to the effect of Mg++. In solutions which contained up to 0.03 molar MgSO₄, the effect of Mg++ predominated over that of SO₄-. Above 0.1 molar MgSO₄, the effect of SO₄- predominated and tended to neutralize the initial deviation. In solutions which contained fixed amounts of MgCl₂ and varying amounts of Na₂SO₄, or vice versa, the effects of these salts on the ionization of oxalic acid antagonized each other in all proportions.

The experimental procedure consisted in determining the pH of solutions of oxalic acid which contained about 1.5 equivalents of NaOH. To these solutions various amounts of MgCl₂ (or Na₂SO₄) and NaCl (or KCl) were added. The pK' values were compared with those of similar solutions having the same ionic strengths but which contained NaCl and no MgCl₂.

These studies were subsequently extended to solutions of gelatin and glycine. The experimental procedure consisted in determining the effects of added salts on the ionic activity of the gelatin or glycine solution. The pH of the 1.25 per cent gelatin solutions lay within the pH range of 7.2 to 7.3.

Simms found that addition of MgCl₂ and NaCl alone to the solution of sodium or potassium gelatinate lowered the pH. Addition of KCl (up to 0.01 M K⁺) first lowered and then raised the pH. Mixtures of NaCl and KCl (up to 0.09 M of the salt whose concentration was varied) raised the pH. As the concentration of Na⁺ or K⁺ was increased to 0.125 M, the pH decreased. Above 0.125 M the mixture of the two salts behaved like KCl. Mixtures of MgCl₂ and NaCl raised the pH, up to 0.10 M Na⁺, and lowered it, up to 0.15 M Na⁺, regardless of the amount of MgCl₂. Higher concentrations of NaCl were found to have no effect. Within this range of NaCl the pH was lowered with increase in the concentration of MgCl₂. Mixtures of MgCl₂ and KCl behaved similarly to those containing MgCl₂ and NaCl. The addition of NaCl plus KCl to the gelatin solution which contained MgCl₂ produced practically the same effect as when either salt was added alone except that the

first two breaks in the curve came at $0.07~\mathrm{M}$ and $0.08~\mathrm{M}$ of Na+ and K+, and the third break came at $0.12~\mathrm{M}$.

The effect of added salts upon the activity of a glycine solution is essentially the same as was found in the case of sodium or potassium gelatinate. Addition of NaCl, KCl, MgCl2, or CaCl2 lowered the pH of a 0.01 M solution of glycine which was half neutralized with NaOH (pH 9.69). The addition of increasing amounts of NaCl up to 0.007 M raised the pH of the glycine solution which contained a given amount of KCl. Further addition of NaCl up to 0.035 M lowered the pH; amounts of NaCl beyond this raised the pH slightly. A similar effect was obtained when NaCl was added to a glycine solution containing MgCl2 or CaCl2 except that the breaks in the curve occurred at 0.015 M and 0.085 M NaCl. When CaCl₂ was added to the glycine solution containing MgCl₂, breaks in the curve occurred at 0.005 M and 0.025 M CaCl2. These concentrations of CaCl₂ correspond, respectively, to ionic strengths of 0.015μ and 0.075μ . The effect is probably a function of the ionic strength of the added salt.

While the experiments of Simms do not explain the entire phenomenon of ionic antagonism, they nevertheless indicate that the underlying mechanism is a chemical one. A hint that proteins may play a rôle in those biological processes in which salt antagonism is noted is contained in Loeb's (5) statement, "The above-mentioned facts of the antagonism between acids and salts suggest the idea that the surface film of cells consists exclusively or essentially of certain proteins."

Work in progress, by Mr. A. C. Batchelder in the writer's laboratory, on the effect of salts on the activities of alanine ions indicates that in this case the effect of added salts on the pH of a 0.03 M alanine buffer, as measured by a hydrogen electrode, is entirely additive. In acidic solutions of pH 2.4 to 2.6, the effects of NaCl, KCl, BaCl₂, CaCl₂, and MgCl₂, up to ionic strengths of 1.0 μ , differ only by amounts that can be accounted for by the Debye-Hückel theory and Kirkwood's extension of it for zwitterions, taking into consideration the different ionic radii. The same is true of mixtures of the above inorganic salts. In alkaline solutions at pH 9.5 to 9.8, MgCl₂ changes the pH by about 0.7 unit at ionic strength 1.0 μ , with CaCl₂, BaCl₂, NaCl, and KCl progressively less, the last altering the pH by less than 0.1 unit. The precipitation of Mg(OH)₂ and the possible formation of undissociated Ca(OH)₂ and Ba(OH)₂, at this pH, are factors which must be taken into

consideration, and which, apparently, were not considered. But here again, the effects are additive in salt mixtures, the slope of the curves representing the effect of the salt whose concentration is varied, being the same as that of the curve obtained with this particular salt alone.

A discussion of ion antagonism in systems which contain inorganic colloids and in biological systems is given by Weiser (7).

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SECTION XV

LIESEGANG RINGS

1. THE FORMATION OF LIESEGANG RINGS

In 1896 Liesegang (1) observed that when several drops of a fairly concentrated solution of silver nitrate were placed on a layer of gelatin gel which contained a solution of potassium dichromate, the precipitate of silver chromate or silver dichromate was not continuous throughout the gel, but formed a series of concentric rings which were separated from each other by clear spaces of gel. These rhythmic precipitates have been termed "Liesegang's rings." It is probable that phenomena of this type were observed by others before their discovery by Liesegang.

An illustration of Liesegang rings is given in Fig. 1. In order to produce the bands it is necessary that the concentration of the solution which diffuses into the gel be higher than that of the electrolyte which is contained in the gel. It is probable that, under proper experimental conditions, rings can be obtained whenever a precipitate results from double decomposition of two reacting electrolytes.

The formation of Liesegang rings is by no means confined to gelatin gels. Bands of cuprous oxide have been obtained by permitting a solution of copper sulfate to diffuse into a silica gel containing glucose. Rings of Turnbull's blue can be obtained in agar gels under suitable conditions. A beautiful illustration of banded precipitation in which two successive reactions take place is the following. Rings of silver chromate are formed by permitting silver nitrate to diffuse into a gel which contains potassium dichromate.

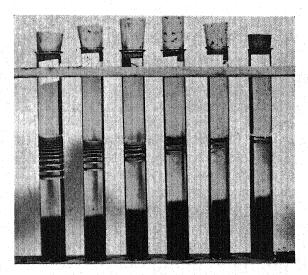


Fig. 1. Liesegang rings. Diffusion of N/10, N/20, N/30, N/40, N/50, N/60, K_2CrO_4 , respectively, into N/100 AgNO₃ in 5 per cent gelatin. (Hughes, E. B., *Biochem. J.*, 28, 1086 (1934).)

After a time the silver nitrate is replaced with lead nitrate. On diffusing into the gel the lead nitrate reacts with the silver chromate to form lead chromate, and silver nitrate is set free. The silver nitrate will diffuse further and form more silver chromate rings which, in turn, are converted into lead chromate.

Rings of precipitated protein are formed when metaphosphoric acid diffuses into a gelatin gel which contains blood serum. Laminated structures are produced when silver nitrate diffuses into wheat grains and the leaves of some plants. The layered concrements seen in gall stones (14) and urinary calculi have been cited as examples of the Liesegang phenomenon. Under suitable experimental conditions the banded structures may be spiral or elliptical in shape. Elliptical rings are formed when silver chromate pre-

cipitates in a gelatin sheet which has been fixed on a sheet of rubber and then stretched.

It is not necessary that the reaction, precipitation, or crystallization takes place in a gel in order to obtain rings. Benzoic acid crystallizes in a beautiful wave-form from a mixture of sulfuric acid and ethyl alcohol. Bands of ammonium chloride are formed when ammonia gas and hydrogen chloride are permitted to come in contact in a long narrow glass tube.

2 THEORIES OF RING-FORMATION

So much has been written and so many attempts have been made to explain the Liesegang phenomenon that lack of space prevents a full discussion here. The literature on the subject has been collected by Hedges (2). No single explanation probably covers all cases of ring formation.

When a suitable electrolyte diffuses into a solution of another electrolyte of lower equivalent concentration, the latter electrolyte generally being contained in a gel, the formation of a banded precipitate depends upon a number of factors. As a result of the diffusion of the entering salt (A) and its reaction with salt (B) contained in the gel, gradients of concentration of both salts are established. When the negative ions of salt (B) react with the positive ions of salt (A) to form a precipitate and are thus removed from solution, more of salt (A) is free to diffuse forward again. The concentration gradient and the rate of diffusion of the entering electrolyte have been put into mathematical terms by Morse and Pierce (3), Stiles and Adair (4), and Fricke (5). Secondly, the formation of rings depends upon the rapid collection of the precipitate into a more closely defined zone than when it first separated. Finally, the phenomenon depends upon the solubility of the precipitate and a factor which may be termed "metastable solubility," or the existence of the precipitate for a time in a condition of supersaturation. The solubility of the precipitate can be altered by the choice of the medium in which precipitation occurs or by the addition of small amounts of a foreign substance to the gel so that it is possible to obtain bands for almost any material which forms as a precipitate by double decomposition of two salts.

Hughes (6) explains the formation of Liesegang rings as follows. Let us assume that X represents the entering ion, Y the ion which is contained in the gel, and that these ions react to form a precipitate XY. We shall represent the external concentration of X by

 C_X , the concentration of Y in the gel by C_Y , the metastable solubility product by S, and the concentration of X required to give the "solubility" product S with C_Y by S_X . The concentrations designated are in excess of the solubility of the product XY. Let the precipitation in the tube of gel occur at a point H_1 . At this point the "metastable solubility product" will have been reached and, since C_Y is approximately constant, S_X for ion X must be constant when precipitation takes place. When precipitation occurs there is a drop in the concentration of the free ions, X becoming zero and Y becoming $C_Y - S_X$. When the amount of X at H_1 and onwards again increases, the amount of Y in excess is precipitated and collected at H_1 so that, as the process goes on, the concentration of Y at point H_1 decreases, while that of X increases. Finally, the concentration of Y at point H_1 reaches the final solubility of XY.

In order that precipitation may occur at point H_2 it is necessary that there be a gradient for Y from C_Y at this point to zero at point H_1 , while that of X differs but little between the two points. The formation of a precipitate at H_2 will take place in accordance with the scheme outlined for point H_1 . The whole procedure is actually a continuous one except for the actual separation of XY when the concentration of $X = S_X$. When separation has occurred the precipitate is concentrated by collection of the particles from the intermediate zones. This is due to the growth of the relatively large particles at the expense of the smallest. This leads to a clearer definition of the bands; it is not the cause of band formation.

The amount of Y which remains between one precipitate and the next is ultimately precipitated so that in order that clear spaces may be obtained it is essential that C_Y should be relatively only slightly greater than S_X . In order that widely spaced bands be formed the gradient of Y must be relatively small. The clearness of the spaces between two bands will depend upon the amount of Y left between a new band and the previous one and hence C_Y should be low. If the gradients of X and of Y are steep, the movement of these ions to the point where precipitation of XY occurs will be rapid and a continuous precipitate occurs. This requires that the concentration of Y be high and that of X considerably higher than that of Y.

According to Jabtczyński (7) the distances between the bands are expressed by

$$\frac{h_n}{h_{n-1}} = K, \qquad \frac{h_n - h_{n-1}}{h_{n-1} - h_{n-2}} = K \tag{1}$$

where h_n , h_{n-1} ... are the distances at which the bands occur, and K is a constant. This equation has been interpreted as indicating that diffusion has a ruling influence on ring formation. The diffusion of the entering ion proceeds so that the distance reached by the free ion is expressed by

$$\frac{h}{\sqrt{t}} = K \tag{2}$$

where t expresses the time during which the diffusing ion has penetrated to a distance, h.

Shemjakin and his co-workers (8) have brought forth evidence which indicates that the distance between bands is governed by the relation

 $\lambda V = K \tag{3}$

where λ = the distance between the rings, V = the velocity of diffusion, and K = a constant for a particular set of conditions. At a suitable concentration of gelatin in the gel the value of K reaches the theoretical constant, k(N/M), where k = Planck's constant, N = Avogadro's number, and M = the molecular weight of the external diffusing salt.

Other discussions of this subject are given by May (9), Bauer (10), Bull and Veil (11), Desai and Nabar (12), and Freundlich (13).

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CHAPTER X

OPTICAL PROPERTIES OF AMINO ACIDS AND PROTEINS

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1. LIGHT ABSORPTION

None of the amino acids and only a few special proteins absorb light in the visible region. However, nearly all of the proteins and certain of the amino acids exhibit a strong specific absorption between 3000 and 2500 Ångström units in the ultraviolet region. This appears to have been first established by Dhéré (1). He attributed the property of the proteins to absorb ultraviolet light to their content of the aromatic amino acids, phenylalanine, tyrosine, and tryptophane, a conception which has, in the main, been substantiated by later investigators.

In those proteins which are visibly colored, the color is not due to the protein moiety, but instead, to some specific colored prosthetic group which is combined with the protein. Important examples of this type of protein are hemoglobin, hemocyanin, and the recently discovered yellow oxidation enzyme of Warburg and Christian (2) in which the chromophore groups are an iron porphyrin, copper, and a flavin pigment, respectively.

Examination of the spectroscopic properties of the different forms of hemoglobin shows that oxyhemoglobin presents two absorption bands, one in the yellow and the other in the green region, while reduced hemoglobin has only one broad band which lies in the yellow green portion of the visible spectrum. Carbon monoxide hemoglobin has a spectrum which is much like that of oxyhemoglobin. Methemoglobin shows a band in the red and is opaque throughout the yellow, green, blue, and violet portion of the visible spectrum.

A very accurate investigation of the absorption spectra of hemoglobin and its derivatives has recently been carried out by Drabkin and Austin (3, 4). These authors determined the absorption curves of reduced and oxidized hemoglobin, of carboxyhemoglobin, nitrohemoglobin, sulfhemoglobin, methemoglobin, and cyanmethemoglobin. They also measured the absorption curves of a number of hemochromogens. Curves illustrating certain of their results are given in Fig. 1. In this figure the light absorption coefficient in terms of both absorption per millimole and per gram

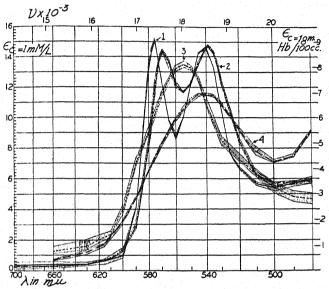


Fig. 1. Absorption curves of HbO₂, HbCO, Hb, and MHbCN, cross-hatched to show the spread of the determinations. Curve 1 represents HbO₂ (eight solutions) 0.070 to 0.205 mM. per liter, buffers 33 mM. per liter of phosphate, 25 mM. per liter of borate (two unbuffered solutions), pH 5.9 to 9.2; Curve 2, HbCO (six solutions) 0.0942 to 0.205 mM. per liter, buffers 33 mM. per liter of phosphate, 10 to 27 mM. per liter of borate (one unbuffered solution and one with 100 mM. per liter of NH₄OH), pH 5.9 to 11.0; Curve 3, Hb (seven solutions) 0.107 to 0.205 mM. per liter, buffers 13 to 27 mM. per liter of phosphate, 24 mM. per liter of borate (two unbuffered solutions), pH 4.5 to 9.2. Reduction was by evacuation in one solution, in the others with Na₂S₂O₄, 4 mM. per liter; Curve 4, MHbCN (seven solutions) 0.113 to 0.205 mM. per liter, buffers 15 to 33 mM. per liter of phosphate, 11 to 25 mM. per liter of borate (one unbuffered solution), pH 5.9 to 9.2. K₃Fe(CN)₆0.8 to 0.9 mM. per liter and KCN 0.7 to 0.8 mM. per liter.

(Drabkin, D. L., and Austin, J. H., J. Biol. Chem., 112, 51 (1935).)

of hemoglobin per 100 cc. of solution is plotted against the wave length and also against the wave number which is the reciprocal of the wave length.

Drabkin and Austin found that a fairly wide variation in pH did not noticeably affect the absorption curves of reduced and oxyhemoglobin, of carboxyhemoglobin, and cyanmethemoglobin. The curves of oxyhemoglobin, carboxyhemoglobin, and nitro-

hemoglobin are very similar, which indicates that oxygen, carbon monoxide, and nitrous oxide form homologous compounds with hemoglobin.

Any agent which causes denaturation shifts the absorption bands of hemoglobin toward the red. The work of Anson and

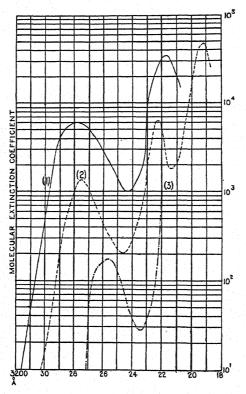


Fig. 2. Ultraviolet spectra of the aromatic amino acids. 1. Tryptophane, 2. Tyrosine, 3. Phenylalanine.

(Smith, F. C., Proc. Roy. Soc. London, 104 B, 198 (1929).)

Mirsky (5) has shown that the absorption spectrum of hemoglobin is that of a hemochromogen. These authors have shown that heme itself does not have a band spectrum. This type of spectrum is produced only when the heme is united to some nitrogen compound thereby forming a hemochromogen. For this reason, the chemical state of the protein moiety of hemoglobin has a marked effect in modifying the resulting absorption spectrum. The spectra of the various hemoglobins are illustrated by Hawk and Bergeim.*

^{*} The absorption spectra of hemoglobin are given by Hawk, P. B. and Bergeim, O., Practical Physiological Chemistry, 11th ed., Philadelphia, 1938.

Among the amino acids, the most marked specific absorption in the ultraviolet region is exhibited by the aromatic amino acids in the descending order of tryptophane, tyrosine, and phenylalanine. The relative magnitudes of their intensity of absorption are about the order of 1.0:0.5:0.05, respectively (6, 7, 8, 9, 10). The ultraviolet absorption curves of these amino acids, reproduced from

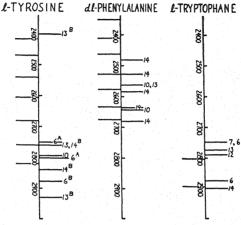


Fig. 3. Absorption bands of l-tyrosine, d, l-phenylalanine, and l-tyrotophane in the ultraviolet region. The horizontal bands to the left of the vertical lines indicate absorption bands found by the authors, while the horizontal bands to the right are what seem to be the most reliable values in the literature. The numbers to the left of the vertical lines are wave lengths in Ångström units, while the numbers to the right of the horizontal lines refer to bibliographic citations. The letters A and B signify that the bands shown for l-tyrosine were obtained in acid (A) or basic (B) solution.

(Feraud, K., Dunn, M. S., and Kaplan, J., J. Biol. Chem., 112, 323 (1935).)

the work of Smith (9), and showing their chief regions of absorption, are illustrated in Fig. 2. The complete absorption spectra of the three amino acids plotted by Feraud, Dunn and Kaplan (10) as line diagrams are shown in Fig. 3. A comparison of the wave lengths of the spectral bands of phenylalanine, tyrosine, and tryptophane, with the bands of the related compounds, benzene, phenol, and indole, also taken from the paper of Feraud, Dunn and Kaplan, is given in Table I.

The specific absorption of phenylalanine, tyrosine, and tryptophane in the ultraviolet region is due to the fact that they are aromatic compounds. It is commonly accepted that the aromatic hydrocarbons are the only ones which exhibit band spectra. The absorption bands of phenylalanine, tyrosine, and tryptophane closely resemble those of benzene, phenol, and indole, respectively, as is shown in Table I. The principal difference between the amino acid and the respective related compound is a shifting of the bands of the amino acids to longer wave lengths.

The nature of the solution has little effect on the absorption of the above three amino acids. Dilute alkali, however, does shift the

Table I

Absorption Bands of l-Tyrosine, Phenol, d,l-Phenylalanine, Benzene, l-Tryptophane, and Indole in Ultraviolet Region

d,l-Phenyl- alanine	Benzene	l-Tyrosine	Phenol	Phenol l-Trypto- phane In	
λ 2675 2643 2576 2525 2462 2410 2350	λ 2685 2645 2605 2545 2480 2420 2380 2330	λ 2820 2760 2680	λ 2760 2690 2625	λ 2894 2804	λ 2873 2790 2710

(Feraud, K., Dunn, M. S., and Kaplan, J., J. Biol. Chem., 112, 323 (1935); 114, 665 (1936).)

points of maxima of the absorption spectrum of tyrosine a little toward the red (8).

The ability of the other amino acids to absorb light is slight in comparison to that of the aromatic amino acids. Feraud, Dunn and Kaplan found that alanine, leucine, histidine, proline, and hydroxyproline showed only a general absorption in the ultraviolet region. According to Gróh and Hanák (8), histidine and cystine exhibit no selective absorption, but show an end absorption at about 2000 Å. The latest investigation on this type of amino acids is by Anslow and Foster (11). They measured the complete absorption spectra of certain amino acids over the wave length range of 6000 to 1850 Å. These authors demonstrated that total absorption of light of short wave lengths occurs in all of the amino acids, and selective absorption in the case of those with symmetrically formed molecules. Examples of their data are shown in Fig. 4. In Table II there are recorded the values of the frequency of maximum

¹ For the infra-red absorption spectra of the stereoisomers of cystine, see Wright, N., J. Biol. Chem., 120, 641 (1937).

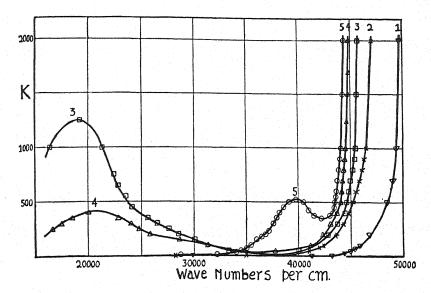


Fig. 4. Variation of molecular absorption coefficients with the frequency of the absorbed light for alanine (Curve 1), cysteine (Curve 2), aspartic acid (Curve 3), glutamic acid (Curve 4), and *l*-cystine in HCl solutions (Curve 5).

(Anslow, G. A., and Foster, M. L.: J. Biol. Chem., 97, 37 (1932).)

selective absorption, the frequency of the region of continuous absorption, and the dissociation energy for a number of the amino acids. Anslow and Foster noted bands of selective absorption for the amino acids in the visible light region of 4500 to 5000Å

Table II

Characteristic Frequencies and Energy of Dissociation

Substance	Molecular weight	Frequency of maximum selective absorption	Frequency of edge of continuous band	Energy of dissociation	
		cm1	cm1	volts	
Alanine	89		49677	6.14	
Cysteine	121		46664	5.77	
Aspartic acid	133	19011	45767	5.66	
Glutamie acid	147	20202	44883	5.55	
Cystine in HCl	240	39841	44425	5.49	
Cystine in H ₂ O	240	39841	45454	5.62	
Butyric acid	88		49950	6.18	
Succinic acid	118	20408	47920	5.81	

(Anslow, G. A., and Foster, M. L., J. Biol. Chem., 97, 37 (1932).)

(see Table II). The explanation offered by these authors is that absorption between 2300 and 2000 Å is due to dissociation of the carboxyl group by the separation of a positive hydrogen from the rest of the groups in the molecule. In the symmetrical amino acids, such as aspartic and glutamic acid, the selective absorption weakens the linkage between the α - and the β -carbon atoms. On

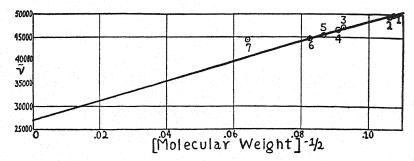


Fig. 5. The frequency of the absorbed light which dissociates the COOH group in (1) butyric acid, (2) alanine, (3) succinic acid, (4) cystine, (5) aspartic acid, (6) glutamic acid, and (7) cystine in HCl solutions, as a function of the reciprocal of the square root of the molecular weight of the absorbing substance.

(Anslow, G. A., and Foster, M. L., J. Biol. Chem., 97, 37 (1932).)

the other hand, the absorption by cystine disrupts the -S-S- group. The disruption of cystine in hydrochloric acid, it is believed, results in the formation of cysteine hydrochloride and cysteine. A plot that shows the frequency of the absorbed light which dissociates the carboxyl group of certain amino acids and related fatty acids as a function of the reciprocal of the square root of the molecular weight is given in Fig. 5.

Considerable attention has been paid to the light absorption properties of the colorless proteins. Measurements have been made of the ultraviolet band spectra of such diverse proteins as casein (8), egg albumin (13), the serum proteins (12, 9), Bence-Jones protein (14), and gelatin (15, 7). As a practical application of this property, Svedberg and his associates, in their work on the determination of the molecular weights of the protein by the ultracentrifuge, have employed the ultraviolet absorption of the colorless proteins as a means of photographically estimating the concentration gradients of protein solutions. The proteins that contain tyrosine and tryptophane show a specific absorption band in the spectral region between 3000 and 2500 Å. This fact is illustrated in the absorption curves of serum albumin solutions, taken from the work of Spiegel-Adolph (12), and shown in Fig.

6. Photographs of the spectra of solutions of serum and egg albumin treated in a variety of ways are reproduced in Fig. 7. Spiegel-Adolph (12) has shown, on irradiating a protein with ultraviolet light or X-rays, that the degree of absorption of ultraviolet light is increased, although the region of absorption is only shifted slightly. This effect is illustrated in Figs. 6 and 7. This alteration in absorption by irradiation is not due to denaturation of the protein, since, as is shown in Fig. 8, heat denaturation does

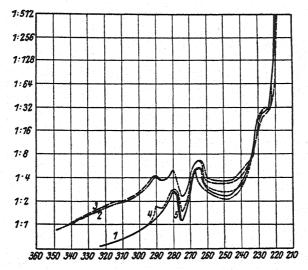


Fig. 6. Absorption curves of solutions of protein: (1) pure serum albumin; (2) irradiated acidified solution of serum albumin; (3) irradiated alkaline solution of serum albumin; (4) control solution of alkaline serum albumin, and (5) control solution of acidified serum albumin. The ratios at the left represent the concentrations of protein; the numerals at the bottom, the wave lengths in millimeters.

(Spiegel-Adolf, M., Archiv. Path., 12, 533 (1931).)

not produce any appreciable alteration in the spectra of serum and egg albumin.

It has been shown by Gróh and Hanák (8) that the region of absorption and the extinction coefficient of a protein can be closely calculated from its known content of tryptophane and tyrosine. The absorption in the proteins is not influenced by acid, but is shifted toward the red end of the spectrum by alkali. The intensity is also increased. This is in agreement with the observed influence of alkali on tyrosine.

Gelatin, which probably contains no appreciable amounts of tyrosine or tryptophane, is found to give only an end absorption in the region between 2500 and 2300 Å. One of the difficulties

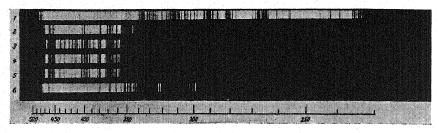


Fig. 7. Influence of ultraviolet rays on the absorption spectrums of solutions of protein (the concentration of protein is the same in all solutions): (1) iron spark; (2) control acidified solution of serum albumin; (3) control alkaline solution of serum albumin; (4) irradiated acidified solution of serum albumin; (5) irradiated alkaline solution of serum albumin, and (6) nonirradiated, electrolyte-free solution of serum albumin.

(Spiegel-Adolf, M., Archiv. Path., 12, 533 (1931).)

involved in working with gelatin is to eliminate the effect of diffuse light dispersion and thus obtain the true light absorption. This difficulty has been overcome by Custers, DeBoer and Dippel (15). The work of these authors shows that the true light absorption of gelatin has the same principal characteristics in gelatin sols as in gels with the exception that the peak at 2500 Å is lower in the gel state. In the range of pH 2.5 to 8.6 the true light absorption of gelatin at 40° is independent of the pH. On the other hand, diffuse dispersion reaches a maximum at the isoelectric point. Gelatin undergoes an optical change on increasing the pH so that a bend which is found in the curve of the spectrum at 2750 Å, at lower pH values, disappears above pH 8.6. According to Custers, DeBoer and Dippel, the light absorption does not depend upon the carboxyl and the amino groups of the protein.

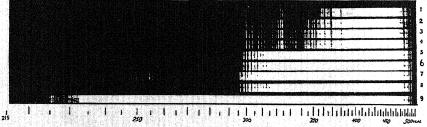


Fig. 8. Influence of heat on absorption spectrums of concentrated solutions of proteins: (1) alkaline solution of serum albumin, boiled; (2) alkaline solution of serum albumin, unheated; (3) acid solution of serum albumin, boiled; (4) acid solution of serum albumin, unheated; (5) alkaline solution of egg albumin, boiled; (6) alkaline solution of egg albumin, unheated; (7) acid solution of egg albumin, boiled; (8) acid solution of egg albumin, unheated, and (9) empty cell.

(Spiegel-Adolf, M., Archiv. Path., 12, 533 (1931).)

2. REFRACTIVITY

The property of refractivity, i.e., the change produced in the direction of a beam of light when it passes obliquely from one me-

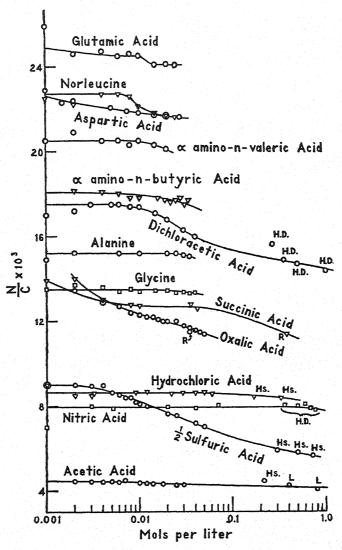


Fig. 9. The relation between N/C and the concentration for solutions of certain acids.

H.D. = Hantzach and Dürigen

Hs. = Hallwachs

L = Lynn

R. = Rimbach

(Craig, R., and Schmidt, C. L. A., Austr. J. Exp. Biol. Med. Sci., 9, 33 (1932).)

dium into another, is based upon the physical fact that the velocity of light depends upon the nature of the medium through which it passes. Since, in a solution, change of temperature, concentration, chemical interaction, dissociation, and association all tend to alter the medium in relation to the passage of light, they will consequently also tend to alter the refractivity of the solution. An extensive study of the influence of the above mentioned variables on the equivalent refractivity of amino acids and proteins has been carried out by Craig and Schmidt (16). In Fig. 9 there are plotted the relationships which they observed between the refractive index and the concentration of the solute of a number of amino acid solutions and, for purposes of comparison, solutions of a number

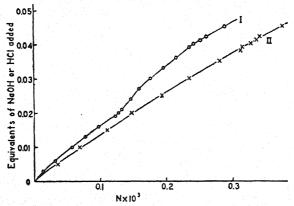


Fig. 10. Titration of aspartic acid. I with NaOH. II with HCl.

Curve I is entirely experimental, since the values for the k_a 's are too close to permit calculation of the theoretical curve. Curve II is theoretical. The crosses indicate experimental values. The relation between the value for N/C for the ion HA-and that for the acid H_2A , is similar to that for glutamic acid.

(Craig, R., and Schmidt, C. L. A., Austr. J. Exp. Biol. Med. Sci., 9, 33 (1932).)

of organic and inorganic acids. The figure shows that values for the equivalent refractivity (N/C) approaches a constant value for each substance as the concentration of the solute decreases. In the symbol N/C, which is used to represent the term equivalent refractivity, N signifies the refractive index of the solution, and C the equivalent concentration of the solute. The results are in good agreement with the deductions which follow from the theory of ionization and the hypothesis that refraction is an additive property. Since, in an extremely dilute solution, ionization becomes complete and the interaction of the ions is reduced to a minimum,

an increase in the refraction and the approach to a constant value may well be expected.

The curves of the very strong acids and of the weak acids show a considerable region of practically constant refraction. Among the divalent acids and acids of intermediate strength, marked changes in the slope of the curves occur. This has been explained by Craig and Schmidt as being due to a change in the degree of ionization. It is also to be noticed that the values of N/C increase

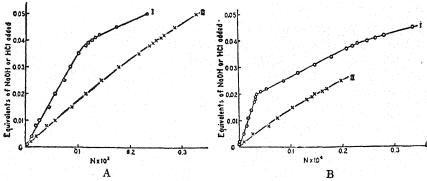


Fig. 11. A, Titration of glycine. I with NaOH. II with HCl. The curves are theoretical. The points are experimental. In Curve II there is no evidence of a break, and even the point of maximum curvature is very difficult to ascertain. B, Titration of arginine monohydrochloride. I with NaOH. II with HCl. The break at 0.02 equivalents of NaOH in Curve I is the transition from the acid salt to the free acid. From this point on, the curve is theoretical. Curve II is also theoretical, and is very similar to the previous curves for the weak base. Curve II embraces only the second k_b for arginine.

(Craig, R., and Schmidt, C. L. A., Austr. J. Exp. Biol. Med. Sci., 9, 33 (1932).)

with increasing molecular weight in a homologous series of compounds.

Since the refractive index of an electrolyte is dependent upon the degree of ionization, it should follow that the course of the neutralization reaction will also be manifested by changes in the refractive index of the solution. Examples for certain amino acids are shown in Figs. 10 and 11, and for gelatin and edestin in Figs. 12 and 13. The gelatinization of gelatin (Fig. 12), it is to be noted, causes a marked alteration in the refractivity curve. The value of N for a solution of isoelectric gelatin was observed to decrease on standing. This is due to the formation of a flocculent precipitate. It forms slowly and separates. The refraction also depends upon the previous history of the gelatin.

The refractivity of proteins has been used as a means for their analytical determination. On the basis of the theoretical equation of Lorenz and Lorentz, $(N^2-1)/[(N^2+2)d] = K$, the relation between refraction and concentration might be expected to be quite complex. In the equation, N is the refractive index, d is the density of the solution, and K is a constant. However, as an approximation

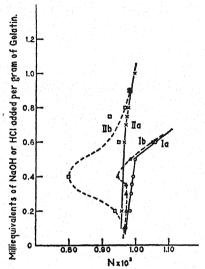


Fig. 12. Titration of gelatin.

I with NaOH

- (a) In the sol condition.
- (b) In the gel condition.
- II with HCl
 - (a) In the sol condition.
 - (b) In the gel condition.

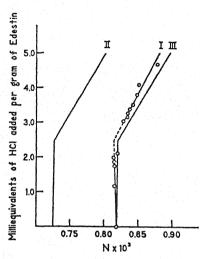


Fig. 13. Titration of edestin.

I Titration of sodium edestinate with HCl.

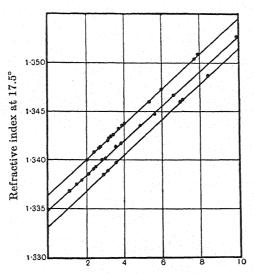
II Titration of sodium hydroxide with HCl.

Curve III is curve II shifted to coincide with curve I, when HCl added equals zero. The value of N included between curves II and III is that part of the total value of N due to the neutralization of the NaOH by the HCl. The dotted portion of I is that range in which the solutions are not optically clear.

(Craig, R., and Schmidt, C. L. A., Austr. J. Exp. Biol. Med. Sci., 9, 11 (1932).)

it has long been known that the equation of Gladstone and Dale (17), (N-1)/d=K, holds quite well. This has led to the conception put forward by Robertson (18), and more recently supported by Adair and Robinson (19), that the refractivity of a protein solution stands in simple proportion to the concentration of the protein. The deduction reached is that the *specific refraction* of a protein is a constant. The specific refraction is defined by the

equation, $\alpha = (N_1 - N_s)/C$, in which α is the specific refraction, N_1 is the refractive index of the protein solution, N_s is the refractive index of the pure solvent, and C is the protein concentration, expressed in terms of grams of dry protein per 100 cc. of solution. It is very interesting, as is pointed out by Adair and Robinson, that the expression for α does not remain constant if the concentration is expressed in grams of protein per 100 grams of



Protein concentration (gm. per 100 cc. solution)

Fig. 14. Refractive indices of protein solutions at 17.5°. Upper curve. Globulin, with 0.19 M KH₂PO₄ plus 0.009 M Na₂HPO₄. Middle curve. Albumin, with 0.0133 M KH₂PO₄ plus 0.0533 M Na₂HPO₄. Lower curve. Albumin dialysed against distilled water.

The lines in Fig. 14 were calculated by the following empirical formulae, in which the first constant equals the refractive index of the dialysate, and the second represents the value of a' or a.

Upper curve R = 1.33639 + 0.001815C.
Middle curve R = 1.33488 + 0.00177C.
Lower curve R = 1.33320 + 0.0018295C.
(Adair, G. S., and Robinson, M. E., Biochem. J., 24, 993 (1930).)

water. The linear relationship between the refraction and the concentration of serum protein solutions is illustrated by the data of Adair and Robinson which are plotted in Fig. 14.

According to Kondo (20) the linear relationship which has been assumed to exist between the concentration and the refractivity of protein solutions is inexact because it neglects to consider the effects produced by changes in ionization due to dilution or alteration of the pH of the solution. However, Craig and Schmidt point

out that the changes in refractivity resulting from alterations in ionization over the physiological pH region are ordinarily a negligible factor.

Adair and Robinson (19) have shown that the specific refractivity of certain amino acids and of the serum proteins can be successfully calculated as an additive function of their chemical composition from the specific refractivities of their constituent atoms.

3. LIGHT SCATTERING (TYNDALL EFFECT)

It is not altogether fortunate that most of the studies on the light scattering properties of protein solutions have been carried out with gelatin, since this property of gelatin, as well as its optical activity, is not typical of proteins in general. In the majority of cases, stable solutions of pure proteins are practically optically clear and scatter but little light.

The work on the light scattering by proteins has been of both a qualitative and quantitative nature. The quantitative interpretation of light scattering is based on the equation of Rayleigh,

$$I = \frac{9\nu\pi^2 v^2 A^2}{\lambda^4 x^2} \left(\frac{N_1^2 - N_2^2}{N_1^2 + 2N_2^2}\right)^2 \sin^2\beta,$$

where I = the intensity of scattered light at a distance x from the scattering particles.

v=the volume of the scattering particle

ν=the number of particles contained in a unit volume

 λ =the wave length of the incident light

A =the amplitude of the incident light

 N_1 = the refractive index of the particles

 N_2 = the refractive index of the solvent

 β =the angle between the incident and the diffracted beams.

If the wave length and the intensity of the incident light are kept constant, and the light scattered is measured at right angles to the incident beam, then the Rayleigh equation may be written as

$$I = K \nu v^2 \left(\frac{N_1^2 - N_2^2}{N_1^2 + 2N_2^2} \right)^2$$

where K now represents a proportionality constant.

The equation of Rayleigh was derived for the case where the particles are small in comparison to the wave length of the light and are assumed to be optically isotropic and distributed at ran-

dom. Some caution therefore needs to be exercised in the application of this equation to proteins where these basic conditions are not always met.

To measure quantitatively the light scattering property of proteins, recourse has been had to such means as photographing the

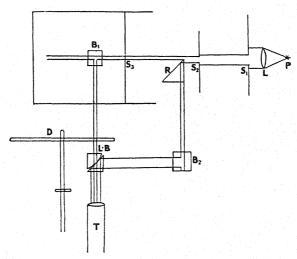


Fig. 15. Apparatus used to measure light scattering.

P is the tungsten arc of a 1000 candle power pointolite lamp; L is a lens used to obtain a nearly parallel beam of light. S_1 and S_2 are circular slits of a suitable diameter placed in the path of the incident light beam so as to cut off any stray light. One portion of the beam that passes through S_1 and S_2 passes through a third slit S_3 , and then through the bottle B_1 containing the material to be tested. The other portion is reflected at right angles to its path by the totally reflecting prism, R. It then passes through the bottle B_2 which contains a solution of a standard material for comparison. Beams of light which are perpendicular to each other and which are scattered by the test material and by the standard are brought into juxtaposition by means of a Lummer-Brodhun photometric head, L.B., and are matched for intensity by means of an Abbey rotating sector photometer, D. T is the observation telescope.

(Krishnamurti, K., Proc. Roy. Soc. London, 129 A, 490 (1930).)

intensity of the Tyndall beam, measurement of the degree of turbidity with a nephelometer, and devices which employ the light intensity differentiating properties of the photoelectric cell. A diagram of the working parts and the description of the photometric apparatus used by Krishnamurti are given in Fig. 15.

Starting with the work of Dhéré and Gorgolewski (21), the light scattering properties of gelatin have been studied by a host of investigators, the principal ones being Arisz (22), Gerngross (23), Vlés and Vellinger (24), Kraemer and Dexter (25), and Krish-

namurti (26). Most of the light scattering effects which characterize gelatin solutions were noted and described by Dhéré and Gorgolewski. Their experimental work was successful because they were the first to use nearly ash free gelatin (about 0.01 per cent ash). These authors noted that purified gelatin in solutions is markedly turbid at temperatures below 30°. They noted further that the addition of acids and bases strongly diminished the turbidity as does also warming to a temperature of over 30°. They also found that the turbidity of gelatin solutions passes through a maximum

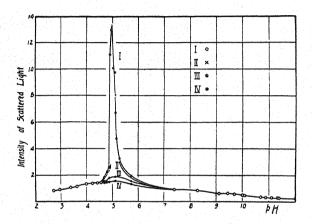


Fig. 16. Influence of pH and temperature upon the relative light scattering capacity of 1 per cent de-ashed gelatin systems.

I—17° II—23° III—30° IV—40° and 50° (Kraemer, E. O., and Dexter, S. T., J. Phys. Chem., 31, 764 (1927).)

with increasing gelatin concentration, the point of maximum turbidity being at about 2.5 per cent.

The main variables which alter the light scattering intensity of gelatin are temperature, concentration of the gelatin, and the pH of the solution. The light scattering capacity of gelatin is most marked at the isoelectric point. In this region a variation of a fraction of a pH unit may cause the light scattering to change by 1000 per cent. This tremendous variation is well brought out in Fig. 16, taken from the work of Kraemer and Dexter. Thus, in one of their experiments, in a solution containing 1 per cent of vacuum dried Eastman's gelatin, the relative intensity of scattered light increased 430 per cent on changing the pH from 4.87 to 4.90, an amount which is but little greater than the experimental error of measurement. If it may be assumed that the maximum turbidity

locates the isoelectric point, then this method is an extremely sensitive means of determining this important constant.

On examining samples of gelatin derived from various sources, Kraemer and Dexter found that the isoelectric region does not fall at the same pH value for all preparations. Most preparations were found to have an isoelectric pH of 5.0, certain bone gelatins were isoelectric at pH 5.5, while a pig skin gelatin gave a maximum intensity of scattering at the high pH of 8.0. These data suggest that gelatin is not a homogeneous substance, but instead, consists of an unknown number of chemical entities. This suggestion is, of course, supported by other types of evidence (see Chapter VII and XVI).

When samples of gelatin which had different pH maxima of light scattering were mixed, there was obtained only a single scattering maximum. It was intermediate between that of the two individual components, and in no instance was a double maxima observed. Thus, on mixing pig skin gelatin having a light scattering maximum at pH 8.0, and calf skin gelatin having a maximum at pH 4.9, in varying proportions, it was found that when the mixture consisted of 50 per cent pig skin and 50 per cent calf skin gelatin, the maximum scattering took place at pH 5.5; with a 10 per cent pig skin gelatin and 90 per cent calf skin gelatin mixture, the maximum scattering occurred at pH 5.9. This value is only slightly different from that of the calf skin gelatin alone. In these mixtures one of the gelatin components usually showed a marked dominance in determining the pH region of maximum scattering.

The increase in turbidity of gelatin solutions produced by a lowering of the temperature is limited to the pH region between 4.5 and 7.5. Below pH 4.5 and above 7.5, a decrease in temperature from 50° to 17° was found by Dexter and Kraemer to produce practically no change in the light scattering. Since stable gels are formed at pH values outside of the above range, it indicates that the turbidity of the gelatin solutions is not linked with the transformation from sol to gel. At pH 3.0, for example, gel formation takes place without any change in Tyndall intensity.

The general form of the curve of the change of turbidity with pH, as shown in Fig. 16, is somewhat asymmetrical, extending more to the basic side than to the acidic side of the isoelectric point.

Hydrogen and hydroxyl ions are the most effective agents in diminishing the light scattering of gelatin solutions. However, it was noted by Gerngross (23) that a decrease in turbidity could be produced, although less effectively, by neutral salts and certain non-electrolytes such as alcohol, urea, and amino acids. The same general behavior as is found for aqueous gelatin solutions was observed by Arisz (22) in the case of solutions of gelatin in glycerol-water mixtures.

A careful study of the relation of temperature and concentration of gelatin to light scattering has been carried out by Krishnamurti

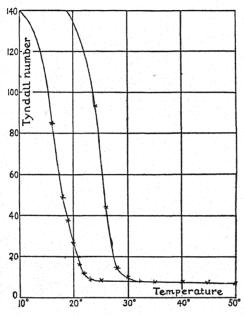


Fig. 17. The influence of cooling and warming on the light scattering properties of ash free gelatin solutions. Cooling curve is on the left, warming curve on the right of the figure.

(Krishnamurti, K., Proc. Roy. Soc. London, 129 A, 490 (1930).)

(26). A plot of the change in the Tyndall number of a 2 per cent purified gelatin solution with change of temperature, which he observed, is shown in Fig. 17. From the figure it may be seen that there is no appreciable change in the intensity of the scattered light as the sol is cooled from 50° to 25°, but below 25° there is a steady increase in turbidity. This variation of the Tyndall intensity is also a function of the time, so that while there is a marked increase as the sol sets to gel, no abrupt change takes place at the setting point. Melting of the gel reverses the turbidity in a manner which is almost identical with that found on cooling. However, a certain lag is observed. In Fig. 17 the cooling curve is given on the

left side, while the melting curve is given on the right side of the figure. On cooling, the intensity changes abruptly between about 25° and 10°, whereas on warming, the abrupt change occurs between the temperatures of 18° and 28°. This difference is very probably only an expression of the time lag which is involved.

It has already been mentioned that the light scattering capacity of gelatin sols depends not only on the temperature, but also on the concentration of the gelatin. This is illustrated by the data

Table III

Effect of Concentration on the Light Scattering Capacity of Gelatin
in the Sol and Gel States

Concentration	Intensity of Scattered Light	Tyndall intensity in gel		
of gelatin per cent	Sol State (40°) Gel State (12°)*	Tyndall intensity in sol		
0.5	1.5 23.1	15.4		
1.0	3.0 50.0	16.7		
2.0	7.5 66.7	8.8		
4.0	7.5 43.0	5.7		
6.0	7.5 17.7	2.4		
8.0	7.5 10.9	1.4		

^{*} The gelatin gel was allowed to set for some hours. (Krishnamurti, K., Proc. Roy. Soc. London, 129 A, 490 (1930).)

which are given in Table III, taken from the work of Krishnamurti. In the sol state the intensity of light scattering increases as the concentration is increased until a value of about 2.0 per cent is reached, beyond which it remains constant. In the gel state the intensity of scattered light passes through a maximum at the concentration of about 2 per cent and decreases again at higher concentrations.

Krishnamurti has also measured the depolarization factor, θ , of the sol-gel change. This term represents the ratio of the intensity of the scattered light which is unpolarized to that which is polarized. According to Rayleigh's theory, the scattered light should be completely polarized in a direction perpendicular to the incident beam. However, this has not been found to be the case for relatively coarse particles. Experimentally, it has been found that the depolarization factor increases with increase in size and diminishes with increasing spherical symmetry of the particles. Thus the term is a qualitative measure of the size and form of the particles. In the work on gelatin it was shown by Krishnamurti that the more the

particles deviate from the spherical shape, the larger does the value of θ become. Illustrative results of Krishnamurti on the alteration in the depolarization factor produced by lowering the temperature of isoelectric gelatin solutions are given in Table IV. At temperatures of 25° or above, the depolarization factor is, within certain limits, independent of the concentration. As the temperature is lowered, θ is increased, the degree of increase being greatest when the concentration of gelatin is 2 per cent or more. In the lower concentrations the factor passes through a minimum as the temperature is decreased. These observations on gelatin are very difficult to incorporate into any single theory of the constitution of gelatin.

Table IV

Variation in the Depolarization Factor of Gelatin with the Transformation from Sol to Gel

	Gelatin Concentrations					
Temperature degrees	2 per cent $ heta$	$\begin{array}{c} 1 \ \text{per cent} \\ \theta \end{array}$	$0.5~{ m per~cent}$ $ heta$			
40	0.038	0.028	0.028			
25	0.038	0.028	0.028			
23	0.034	0.031	0.034			
21	0.038	0.031	0.028			
20	0.045	0.028	0.025			
18	0.072	0.031	0.020			
15	0.198	0.082	0.025			
10	0.455	0.180	0.053			

(Krishnamurti, K., Proc. Roy. Soc. London, 129 A, 490 (1930).)

In general, it may be stated that in the sol state above 30°, the gelatin micelles are well defined and are highly dispersed. Furthermore, the changes which result from a change in pH, in temperature, or in concentration, take place fairly rapidly and reversibly. In the viewpoint of Krishnamurti, gelatin sols above 30° are polydisperse systems in which at least part of the gelatin is molecularly dispersed. On cooling a dilute solution of isoelectric gelatin, the system becomes supersaturated and this leads to the formation of molecular aggregates. The particles thus formed become larger due to the further condensation of gelatin. The steep rise in Tyndall intensity in the neighborhood of the isoelectric point indicates a strong tendency of gelatin to precipitate from a solution which has become supersaturated. In corroboration of this theory, Krishnamurti offers the following evidence obtained by ultramicroscopic

examination with the cardiod ultramicroscope. Gelatin sols at about 30° show no individual particles. As cooling proceeds, a large number of very fine particles of different sizes, which are in rapid Brownian motion, become visible. Gradually the smaller particles aggregate to larger ones, and, with increase in size, the Brownian motion finally ceases.

It is not readily apparent how the fact that the turbidity of gelatin passes through a maximum with increasing concentration of gelatin is to be fitted to this theory. The assumption that there is a true solubility supersaturation of the gelatin is not easily maintained. In the estimation of Kraemer and Dexter, the effect of lowering the temperature on the light scattering behavior of gelatin is more in harmony with the conception that there is a critical peptization temperature for gelatin, or that it forms a conjugate solution with a consolute temperature.

A study of the scattering of light by protein solutions more typical than gelatin has been carried out by Putzeys and Prosteaux (27). These authors started out with the object of testing whether the intensity of scattered light was simply related to the molecular weights of the proteins. From the measurements of Svedberg (28) the proteins, ovalbumin, amandin, excelsin, and hemocyanin (of *Helix pomatia*), with the molecular weights of 34,500, 208,000, 212,000, and 5,000,000, respectively, were chosen as test substances. The comparison was based on the light scattering equation of Rayleigh which has already been discussed (page 566). From this equation it can be deduced that, under certain conditions, the ratio of the light scattered by a series of compounds is proportional to their molecular weights.

In the protein series studied by Putzeys and Prosteaux, the refractive index of each member had nearly the same value. The depolarization factor could be neglected, as all of these protein molecules are nearly spherical. Introduction of these conditions leads to simplification of the Rayleigh equation to the form,

$$I=K\nu v^2,$$

in which ν represents the number of particles per unit volume and v their volume. It is readily apparent that the term, νv^2 , is proportional to the molecular weight and the concentration of the protein solution.

The light scattering results obtained by Putzeys and Prosteaux are given in Fig. 18, in which the value of the proportionality con-

stant of the Rayleigh equation is plotted against the concentration of protein per cc. for the various wave lengths of light employed. In Fig. 18 there are plotted the light scattering when the total light of a sodium flame was used, and the scattering produced with light of wave lengths 5461 and 5769-5790 Å. The authors point out that the proportionality constant, K, need not be expected to maintain a fixed value totally independent of the protein concentration of the solution. However, as the protein concentration decreases, the constant should approach a limiting value, K_0 , at

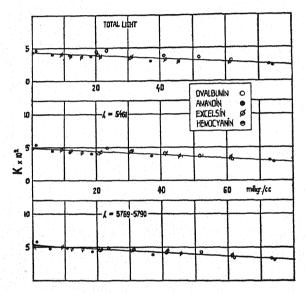


Fig. 18. The light scattering properties of certain proteins as a function of their concentration.

(Putzeys, P., and Prosteaux, J., Trans. Faraday Soc., 31, 1314 (1935).)

infinite dilution, which is a true constant valid for all proteins. The data of Fig. 18 support this viewpoint. From the figure it is clear that, at each wave length, all of the proteins converge toward a single value of K_0 . Moreover, the points obtained for change of the values of K of the proteins, ovalbumin, amandin, and excelsin, with increasing protein concentration, fall on the same straight line. For light of the yellow doublet, the average divergence of the points from the same straight line is: zero for excelsin, +3.2 per cent for ovalbumin, and -4.6 per cent for amandin. Only a few points obtained with hemocyanin are plotted. Due to its great size, the extension of the graph to cover its range was impracticable.

In the above work neither the pH nor the salt concentration of the protein solutions was definitely fixed. The pH of the ovalbumin solution was 4.6; amandin, 5.54; excelsin, 6.9; and hemocyanin, 5.85. The salt content of the different protein solutions varied from half saturation with ammonium sulfate to that of a dilute phosphate buffer. From the results obtained it may be concluded that the main factor which determines the light scattering of an isotropic protein solution is the molecular weight of the protein. Moreover, its scattering power is a true measure of its molecular weight. Such influences as hydration and ionization must necessarily play an insignificant rôle.

The Raman spectra of certain amino acids have recently been measured by Edsall (29). The spectra were found to be closely related to the acids and the amines which are homologous with the amino acids. According to Edsall, the work offers support for the zwitterion theory of amino acid structure.

4. OPTICAL ROTATION

The unit most commonly used to characterize the optical rotatory power of a compound is the specific rotation. This is calculated from the polariscopic reading according to the formula,

$$(\alpha)_{\lambda}^{t^0} = \frac{100\alpha}{adl},$$

in which $(\alpha)_{\lambda}^{l^0}$ is the specific rotation at the wave length, λ , and temperature, t, α is the observed angle of rotation, l is the length of the polarimetric tube in decimeters, d is the density of the solution, and g is the number of grams of the substance under test per 100 grams of solution. In other words, the specific rotation of a substance is the rotation expressed in degrees which is given by 1 gram of substance when it is dissolved in 1 cc. of water in a tube 1 decimeter in length.

For accurate polariscopic measurements a monochromatic source of light is required. The bulk of the measurements in the past have been carried out with the sodium flame. This light source is difficult to obtain with a constant intensity of illumination and is not sufficiently monochromatic for very precise measurements. For this reason, since the development of the mercury vapor lamp, which more nearly approximates the ideal requirements, the green line of mercury vapor ($\lambda = 5461$ Å) is now largely used as a standard for accurate polarimetric work.

Excepting for glycine, all of the known amino acids contain one or more asymmetric carbon atoms each and, as a consequence thereof, are optically active. Since proteins contain asymmetric amino acids, they are therefore optically active.

Of the influences which affect the optical rotation of amino acids and proteins, among the most important is the acidity or alkalinity

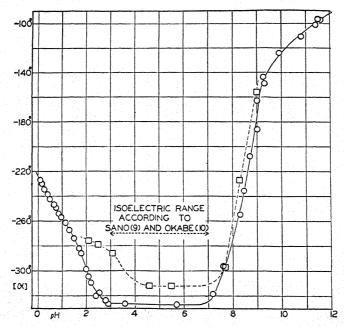


Fig. 19. Specific rotation of l-Cystine plotted against pH. Cystine, 0.005 M. Average temperature, $30.0^{\circ}\pm0.5^{\circ}$ for pH 1.1 to 11.6 and $31.0^{\circ}\pm0.5^{\circ}$ for pH 0 to 1. pH values between 0 and 1 are calculated from the HCl concentration (0.14 to 1.02 M) according to the activity coefficients of HCl the cystine concentrations being disregarded, while above 1 the pH was estimated colorimetrically. For the determinations below pH 1 free cystine was used instead of the Li salt. The broken line curve refers to the data of Pirie for cystine 0.0083 M, temperature 20°.

(Toennies, G., Lavine, T. F., and Bennett, M. A., J. Biol. Chem., 112, 493 (1936).)

of the solution. The influence of pH on the optical rotation of an optically active electrolyte, with the resulting changes in dissociation, has been extensively studied. Usually the specific rotation of the neutral molecule differs from its free ions, and the observed rotation in solution is an additive function of the fractional values of the component molecular and ionic species which are present, so that, in many instances, it has been possible to correlate closely the rotation curves with the dissociation constants of the

substance. However, attempts to forecast the optical rotatory power of an organic compound from a study of its structural formula have generally been abandoned, since the problem has proved to be too complex to be solved in any simple manner.

To a considerable degree a relation between the optical rotation and the acidic and basic properties of the amino acids appears to exist, although no very precise data are available on this point. A more recent illustration of the relation of the optical rotation of an amino acid to the degree of neutralization and pH is furnished by measurements on *l*-cystine (30) (see Fig. 19). In general, the observed rotatory relationships found among the proteins are far too complex to be fitted into such a simple scheme.

The most complete series of measurements of the rotatory power of amino acids in solutions of acid and alkali have been carried out by Lutz and Jirgensons (31). These authors used the curves which they obtained as a means of deciding whether a given naturally occurring amino acid belongs to the d- or l-configuration. This was done by comparing the curves with that of an amino acid of known configuration. The amino acid test standard used for this purpose and whose configuration relationship had been established by synthetic means was aspartic acid. In carrying out the test, the acid branch of the optical rotation curve was found to be most characteristic. If, with increasing acid concentration, it is found that the rotation changes toward the positive direction, then, according to this test, the amino acid is the l-antipode, while, if the rotation becomes more negative, the amino acid is the d-antipode. This is illustrated by the optical rotation curves of the amino acids, taken from the data of Lutz and Jirgensons, and reproduced in Fig. 20. The curves in this figure represent specific rotations measured at the wave length of the D line of sodium. Most of the measurements were made in concentrations of 0.05 molal amino acid and at 20°. The exceptions to this are noted in the legend to the figure. The abscissa values of HCl and NaOH represent the ratios of the moles of acid and base to the moles of amino acid present in the solution.

For purposes of identification, and as a test of purity, measurement of the rotation values of the amino acids in hydrochloric acid is a standard procedure. It should be noted that, for this purpose, unless the values for the concentration of amino acid and hydrochloric acid are closely specified, the data may be inaccurate. The work of Lutz and Jirgensons shows that the actual concentration of amino acid as well as the HCl to amino acid ratio has a

marked determining effect on the magnitude of the specific rotation. Thus, to cite specific examples, at the same HCl ratio, 0.3 M aspartic acid has a 2.0° higher specific rotatory value than 0.1 M, 0.2 M hydroxyproline has a 2.3° lower rotation than 0.05~M, 0.2 M lysine dihydrochloride has a 1.3° higher rotation value than 0.05~M.

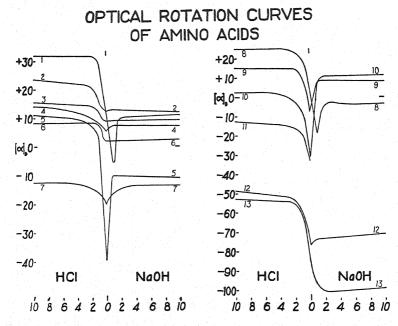


Fig. 20. Optical rotation curves of amino acids in acid and alkaline solutions. Legend: The ordinate represents the specific rotation of the amino acids. The abscissa gives the ratio of the number of moles of HCl and NaOH, respectively, to the moles of acid in the solution. The numbers refer to the following amino acids: 1.0.01 M glutamic acid; 2.0.05 M arginine; 3.0.05 M lysine; 4.0.05 M ornithine; 5.0.05 M histidine; 6.0.02 M alanine; 7.0.05 M tyrosine; 8.0.01 M aspartic acid; 9.0.01 M leucine; 10.0.05 M tryptophane; 11.0.05 M dioxyphenylalanine; 12.0.05 M hydroxyproline; 13.0.05 M proline. The determinations of glutamic acid, tyrosine, and aspartic acid were carried out at 18°, and the others at 20°.

(Lutz, O., and Jirgensons, B., Ber. chem. Ges., 63, 448 (1930); 64, 1221 (1931).)

If salts are present they may have a marked influence on the optical rotation. Pfeiffer (32) long ago observed that neutral salts affected the optical rotation of certain amino acids. He assumed this to indicate combination between the salt and the amino acid. The optical rotation of all amino acid solutions, however, is not influenced by salts. Liquier-Milward (33) found little salt effect on the optical rotation of d-alanine or of d-aspartic acid. On the other hand, this same author observed a strong influence of neutral

salts on the rotation of asparagine. The results which she obtained with this amino acid amide are reproduced in Fig. 21. The measurements were carried out on a 2 per cent asparagine solution at the wave length of the light obtained from the mercury arc. The specific effect of each salt was compared, as is shown in Fig. 21, by plotting the optical rotation against the square root of the ionic strength of the salt solutions. From this work the alkali halides were found to vary the rotatory power in a nearly linear fashion

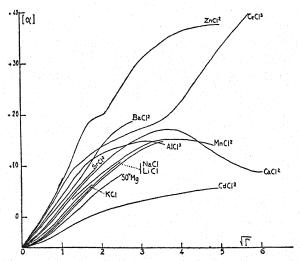


Fig. 21. The influence of neutral salts on the optical rotation of asparagine. (Liquier-Milward, J., Trans. Faraday Soc., 26, 390 (1930).)

in the order given by the series KCl < NaCl < LiCl. In general, strong electrolytes have a more marked influence than weak electrolytes, e.g., CdCl2, which is a weak salt, exerted the least influence.

Liquier-Milward explains the altered rotatory power of asparagine which is produced by salts on the hypothesis that the salts induce a polarization of the molecule or, in other words, tend to shift the structure from the neutral molecule to the zwitterion form. In support of this interpretation the evidence is cited that in strongly acid solutions of asparagine, neutral salts produce no change in the optical rotation because now the amino group already has a fixed hydrogen ion and is no longer subject to further polarization.

A compilation of the optical activities of amino acids and related compounds is contained in Table V.

Table V
Optical Activities of Amino Acids¹

Amino Acid	$Solvent^3$	Temp.	$[\alpha]_D$	Ref.
d-Alanine	M/5 in water	20	+1.0	1
	10% in water		+2.1	6
	M/5 (1 mole A.A.+15 M HCl)	20	+9.7	2a, (2c)
	6.2% in N/1 HCl	20	+9.7	3a
	8.6% A.A. hydrochloride in water	20	+9.6	4, (10)
	10.2% A.A. hydrochloride in water	20	+10.3	5a, (11)
l-Alanine	3.6% in water		-2.5	6
	7% A.A. hydrochloride in water	20	-10.3	5a
	9% A.A. hydrochloride in water	20	-9.8	7
	9.3% A.A. hydrochloride in water	20	-9.7	4a
d-Arginine	M/20 in water	20	+12.2	1
	M/5 in water	20	+12.5	1
	M/20 (1 mole A.A. +10 M HCl)	20	+23.5	1
	M/5 (1 mole A.A.+10 M HCl)	20	+24.1	1
	8.1% in water	20	+12.9	8
	1 mole A.A. in 8 moles HCl	20-25	+26.5	9
d-Aspartic acid	4.2% in 3 M HCl		-25.5	4a, (4b)
l-Aspartic acid	M/10 in water	18	+4.7	2a, (2b), (2c), (47)
	M/10 (1 mole A.A. +10 moles HCl)	25	+25.0	2a, (4a)
	1 M in 3 moles HCl	25	+25.1	10, (2b)
	9.1%+3 moles HCl	20	+26.4	11
l-Cysteine	1 M in 1 M HCl	26	+9.72	12
	1 M (12.1%) in 1 M HCl	26	+7.6	12
d-Cystine	HCl	20	+221	13
l-Cystine	1% in 1 N HCl	31.5	-200²	14
	0.4% in 1 N HCl	31.5	-198.5	14, (48b)
	M/20 (1 mole A.A. +4 moles HCl)	18.5	-213.3	2a
l-Dihydroxy-	10.9% in 1 N HCl	20	-14.3	15
phenylalanine		20	-19.1	1

¹ For method of determining optical rotation see Lowry, T. M., Optical Rotatory Power, London, 1935. See also Freudenberg, K., Stereochemie, Leipzig and Vienna, 1933.

² [α]₅₄₆₁.

³ The concentrations in this table, when expressed in per cent, are approximate. For more exact values, consult the references.

A.A. = Amino Acid. In each instance this refers to the amino acid given in the first column.

Reference in () indicates additional reference, the data from which are not cited.

TABLE V (Continued)

Amino Acid	$Solvent^3$	Temp. (degrees)	[α]D	Ref.
d-Diiodotyro-				
sine	4.8% in 4% HCl	20	+2.9	16
l-Diiodotyro- sine	5% in 4% HCl	19	-2.9	17
d-Glutamic	M/10 in water	18	+11.5	2a, (2b)
acid	M/10 (1 mole A.A. +10 moles HCl)	18	+32.0	2a, (4a)
aora	5.7% in 20% HCl	20	+31.9	18
	1 mole A.A. in 4.53 moles HCl	25	+32.2	10, (2b)
	1.269 gm. A.A. +0.2483 gm. HCl in	20	, 0	-0, (-0)
	25 cc. water	20	+31.1	11
7 Clutomia	407 in water	26	-12.9	19
l-Glutamic	4% in water	20	-30.0	20, (4a)
acid	5.3% hydrochloride in water	20	-30.0	20, (44)
d-Histidine	2.3% in water	20	+40.2	21
W 111501011110	3.2% in water	20	+39.3	20a
	5.3% hydrochloride in 3 N HCl	20	-7.9	20a
<i>l</i> -Histidine	M/20 in water	20	-39.3	1
	M/20 (1 mole A.A. +10 moles HCl)	20	+11.1	1
	2.2% in water	20	-39.7	21
	2.3% in water	20	-37.9	20
	3.1% in 1 N HCl	18	+9.6	22
1 O TT			108	23
d - β -Hydroxy-	4% in water		+0.8 +16.3	23
glutamic acid	2% in 20% HCl		710.0	20
d-Hydroxy-	Water	20	+75.2	24
proline	Water	18	+58.6	24
l-Hydroxy-	M/20 in water	20	-76.3	1
proline	M/10 in water	20	-75.9	1
prome	9.3% in water	20	-81.0	25, (24)
		20	-80.6	26
	12.8% in water	20	-59.5	1
	M/20 (1 mole A.A.+1 mole HCl) M/10 (1 mole A.A.+10 moles HCl)	1	-47.3	1
				o# (00)
d-Isoleucine	3.7% in water	20	+9.6	27, (28), (52)
	4.54% in 20% HCl	20	+36.7	27, (10),
				(20b)
<i>l</i> -Isoleucine	3.9% in water	20	-12.7	20a, (52)
- Indiduding	4.2% in 20% HCl	20	-40.9	28
	4.6% in 20% HCl	20	-40.1	20a

TABLE V (Continued)

Amino Acid	${f Solvent^3}$	Temp.	$[\alpha]_D$	Ref.
d-Leucine	2.1% in water	20	+10.3	7
	3.66% in 20% HCl	20	-15.6	29
	3.66% in 20% HCI	20	-15.4	7
	4.73% in 20% HCl	20	-17.5	30
l-Leucine	M/10 in water	20	-7.2	2a, (2b)
	2.25% in water	20	-10.7	27
	M/10 (1 mole A.A. +30 moles HCl)	20	+16.0	2a, (2b)
	3.5% in 1 N HCl	18	+15.6	22
	3.65% in 20% HCl	20	+15.4	27
	4.62% in 20% HCl	20	+15.9	29
	4.9% in 22% HCl	20	+18.6	31
	7% in 20% HCl	20	+15.7	10
d-Lysine	M/20 A.A. dihydrochloride in water	20	+14.1	1
	M/20 (1 mole A.A. +8 moles HCl)	20	+15.5	1
	Water	20	+14.6	32
	8 N HCl		+15.5	1
l-Methionine	5% in water	20	-7.3	33
	2.78% in water	20	-7.2	34
d-Norleucine	9.2% in water	20	+5.2	35, (29)
	9.8% A.A. hydrochloride in water	20	+16.0	35
	4.6% in 20% HCl	20	+23.1	42
<i>l</i> -Norleucine	9.7% in water 11.6% A.A. hydrochloride in 20% HCl	20 20	-4.5 -16.1	35, (38) 35, (42)
		20	10.1	00, (42)
d-Phenylal-	2% in water	16	+35.1	36
anine	2% in water	20	+35.0	37
	2% in water	20	+34.6	27
	3.5% in 18% HCl	20	+7.1	36
	3.5% in 20% HCl	20	+6.9	27
l-Phenylal- anine	1.9% in water	20	-35.1	37
d-Proline	5.2% in water	20	+81.9	38
	3.9% in water	20	+81.5	38
<i>l</i> -Proline	M/20 in water	20	-86.9	1
	4.4% in water	20	-79.8	38
	6.5% in water	20	-80.9	38
	8.1% in water	20	-84.9	26
	M/20 (1 mole A.A. +10 moles HCl)	20	-52.6	1
	7.2% in 20% HCl	20	-54.5	26

Table V (Continued)

Amino Acid	$Solvent^3$	Temp. (degrees)	$[\alpha]_D$	Ref.
d-Serine	10% in water	20	+6.9	39
	10% in water	20	+6.7	27
	8.9% in N HCl	20	-14.3	39
	8.9% in N HCl	20	-14.1	27
l-Serine	10% in water	20	-6.8	39
	10% in N HCl	25	+14.5	39
d-Thyroxine	0.74 gm. A.A. in 6 gm. 0.5 N NaOH and 14 gm. alcohol	21	+3.02	40
l-Thyroxine	0.66 gm. A.A. in 6.07 gm. 0.5 N			
t-1 hyroxine	NaOH and 13.03 gm. alcohol	21	-3.2^{2}	40
	4.8% in N HCl	23	-12.0	40
d-Tryptophane	0.5% in water	20	-32.5	41
l-Tryptophane	M/20 in water	20	-31.3	1, (48b)
	0.5% in water	20	-32.1	41
	M/20 (1 mole A.A.+1 mole HCl)	20	-7.8	1
d-Tyrosine	4.6% in 21% HCl	20	+8.6	43
	4.8% in 1 molar HCl	23	$+12.5^{2}$	44
l-Tyrosine	M/20 (1 mole A.A.+3 moles HCl)	20	-13.6	2a
	4.7% in 4% HCl	20	-13.2	43
	4.8% in 1 molar HCl	23	-12.4^{2}	44
	5% in 4% HCl	21	-16.1	10
	5% in 4% HCl	16	-16.1	45
d-Valine	3.6% in water	20	+6.4	49
	3.2% in 20% HCl	20	+28.7	49
<i>l</i> -Valine	5.4% in water	18	-5.7	7
	6.2% in water	20	-6.1	27
	4.6% in 20% HCl	20	-27.6	27
	4.6% in 20% HCl	20	-27.4	7
	Miscellaneous			
d - α -Amino- n -	5.4% in water	20	+8.0	36, (51)
butyric acid	5.0% A.A. hydrochloride	20	+14.5	36
l - α -Amino- n -	5.3% in water	20	-7.9	36
butyric acid	4.8% A.A. hydrochloride	20	-14.3	36
l-Asparagine ·	M/10 in water	20	-5.3	46 (47)
H_2O	2.0% in water	25	-5.5	47

TABLE V (Continued)

Amino Acid	Solvent ³	Temp.	$[lpha]_D$	Ref.
	M/10 (1 mole A.A.+10 moles HCl) 10.2% in 10% HCl	20 20	+28.3 +27	46, (2c)
l-Canaline	1.6% in water	21	-8.1	50
d-Isoleucine	2.4% in water	20	-14.4	48a, (52)
(allo)	2.9% in water	20	-14.2	48a
	3.6% in water	20	-12.9	27
	4.6% in 20% HCl	20	-36.8	48a
	4.7% in 20% HCl	20	-34.4	27
<i>l</i> -Isoleucine	Water	20	+14.0	48a, (52)
(allo)	20% HCl	20	+38.1	48a
d-Ornithine	M/5 A.A. dihydrochloride M/20 (1 mole dihydrochloride+8	20	+13.3	1
	moles HCl)	20	+14.1	1
l-Valine (iso)	5% in water	20	-9.1	27
	3.7% in 20% HCl	20	-6.1	27

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Returning to a consideration of the optical properties of the proteins, it is found that, with the exception of gelatin which is a very atypical protein, there are but few data available on their rotatory behavior. The influence of pH on the optical rotation of certain proteins has been investigated by Almquist and Greenberg (34), Pauli and co-workers (35, 36, 37), Jessen-Hansen (38), and Haugaard and Johnson (39). A good deal of this work was carried out over only a restricted range of pH. Jessen-Hansen and Hau-

gaard and Johnson found a nearly linear relationship for ovalbumin and gliadin solutions in the pH range of the isoelectric region. On the basis of these measurements, Abramson and Grossman (40) have postulated that the optical rotation of a protein varies with the pH in a manner which is directly proportional to the mobility of the protein ions. So far this hypothesis has not been subjected to a thorough test.

The aim of Almquist and Greenberg, in investigating the influence of acid and alkali on the change of rotation of proteins, was

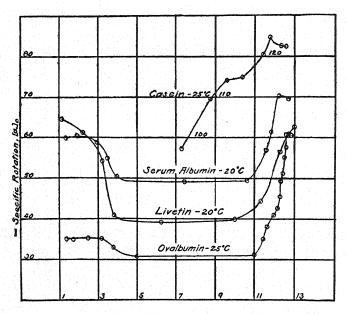


Fig. 22. Effect of pH on change of specific rotation of several proteins. (Almquist, H. J., and Greenberg, D. M., J. Biol. Chem., 105, 519 (1934).)

to obtain evidence on the mode of combination of proteins with acids and bases. The rotation curves of approximately 1 per cent solutions of ovalbumin, livetin, serum albumin, and casein were determined by using the D line of sodium as a light source. On the addition of either acid or alkali, the optical rotation of these protein solutions at once attained a constant value, which could be immediately restored to the original value by neutralizing the solution with an equivalent amount of alkali or acid.

The curves obtained by these authors are reproduced in Fig. 22. From this figure it may be seen that the rotation curves, particularly those of serum albumin, livetin, and ovalbumin, show a con-

siderable degree of similarity. There is a minimum zone of rotation for each protein which extends through the isoelectric region and covers the range of about pH 4.5 to 10.0. On both the acidic and alkaline side of this minimum zone, the levo-rotation increases steeply until maximum values are reached. While similar in form, the curve of each protein is specifically different in magnitude.

Pauli and Kölbl (36) have published similar curves for the optical rotation of ovalbumin and serum albumin solutions. The general forms of the curves obtained by these authors are in good agreement with those of Almquist and Greenberg. However, the initial rotatory values of their preparations of ovalbumin and serum albumin were found by Pauli and Kölbl to be about 6° more levo-rotatory in each case. The authors attribute this difference to their having prepared more highly purified, electrolyte-free proteins than did Almquist and Greenberg.

Since a large body of work points to the uncombined basic groups of the amino acids, arginine, lysine, and histidine, as being the source of the acid-combining capacity, and the uncombined acidic groups of tyrosine, aspartic, glutamic, and β -hydroxyglutamic acid as being the source of the base-combining capacity of proteins in aqueous solution, it would appear to follow logically that the optical rotation of a protein should reflect the rotation found among these amino acids under the influence of pH. However, a comparison of the rotatory curves of the amino acids, given in Fig. 20, with those of the proteins, given in Fig. 22, shows that the protein curves can be explained only partially in terms of the effect to be expected from the basic and acidic amino acids. The correlation with the dicarboxylic acids is in the right direction. On the other hand, the behavior of the basic amino acids offers no analogy for the rotational change of the proteins on the acid side of the isoelectric point. In acid solutions, lysine and arginine show only a small rotational change and histidine a very large one, but in all cases the direction is toward the dextro rather than the levo side, as is found with the proteins.

The changes in optical rotation produced by heat denaturation have recently been studied by Barker (41) and by Pauli and coworkers (36, 37). According to Barker, the rotatory power of alkaline, heat-denatured egg albumin varies in an orderly manner with the time and the temperature of heating. The optical rotation approaches a definite limiting value which is primarily a function

of the pH and the protein concentration of the solution during the actual period of heating.

A large amount of study has been devoted to the rotatory properties of gelatin (42, 43, 44, 45, 46, 47). This has brought out the fact that gelatin has a unique behavior and does not show the variation with pH common to the other proteins. According to Kraemer and Fanselow (44), the change in rotation produced by pH or temperature cannot be correlated with influences arising from dissociation. On the other hand, that a strong parallelism exists between the changes in optical activity and gel formation is clear from the work of Smith (42) and of Kraemer and Fanselow (44). At and above 35°, the specific rotation is practically constant and independent of the concentration of gelatin, pH, temperature. and the presence of specific electrolytes. However, cooling to temperatures below 30° leads to a large increase in the levo-rotation of the gelatin. This continues for several days, when the temperature is held constant, before a steady value is reached. Below 15° the change in rotation which is produced by still further lowering the temperature again becomes slight. In the opinion of Smith, the rotatory changes in the region of 30° to 15° are due to mutarotation. The phenomenon is reversible. The rotation is readily reversed in either direction by changing the temperature.

This behavior of gelatin led Smith to postulate the existence of two forms of gelatin which are in thermal equilibrium with each other, one existing in the sol and the other in the gel state, each form being characterized by a distinct specific rotation value. Smith estimated that the sol form has a specific rotation of -140° at 35° and the gel form -313° at 15° at the wave length of sodium light. The sol form is stable only at temperatures of 30° or over, the gel form at temperatures of 15° or less. Since the mutarotation on lowering the temperature was found to follow the course of a second order reaction, the explanation was proposed that two molecules of the sol form combine to produce one molecule of the gel form of gelatin.

Kraemer and Fanselow observed the presence of optical rotation maxima at the pH values of 1, 5, and 8.5 at temperatures below 30°. At low gelatin concentrations, the rotation maxima and minima are very pronounced, while at higher concentrations they tend to disappear.

Any agency which prevents the appearance of a high levo-rotatory change in gelatin also inhibits its setting to a gel. This may

be an irreversible or reversible effect. Heating gelatin for a few minutes at 140° is stated by Smith to destroy irreversibly the mutarotation and the power of gelatin to form a gel. The chief reversible agents are acid, alkali, and certain neutral salts. The last have a strong influence on the optical rotation of gelatin, particularly at gelatinizing temperatures.

The influence of neutral salts on the optical rotation of gelatin has been extensively studied by Carpenter and his co-workers (45, 46, 47). The salts of weak organic acids have little influence on the rotation. Thus the potassium salts of formic, acetic, and pro-

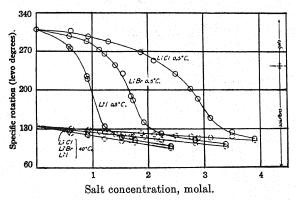


Fig. 23. Influence of lithium halides on the specific rotation of gelatin. (Carpenter, D. C., and Lovelace, F. E., J. Amer. Chem. Soc., 67, 2337 (1935).)

pionic acids were found to exert little effect at either 40° or 0.5°. On the other hand, the alkali halides and thiocyanate exert a very powerful influence, particularly at the lower temperature. The curves obtained by Carpenter and Lovelace for the influence of the lithium halides on a 0.77 per cent solution of calf skin gelatin at the pH range of 6 to 7 are illustrated in Fig. 23. A similar. though somewhat less marked influence, is exerted by the halides of the other alkaline elements. The capacity of these salts to decrease the levo-rotation of gelatin is in the order CNS⁻>I⁻>Br⁻ >Cl-. This indicates that their influence on the optical rotation of the gelatin follows a Hofmeister series. The effect on the rotation of these salts is paralleled by their effect in inhibiting the formation of gelatin gels. The effects of these electrolytes are reversible so that when the solutions were dialyzed free from salt the gelling property of the gelatin solutions returned, and the optical rotation was restored to its initial value. The change in optical activity, as will be considered in more detail below, may be attributed to a tautometric equilibrium between two forms of gelatin which characterize the sol and gel states.

Certain other reagents which are termed by German colloid chemists hydrotropic substances may have a marked effect on the specific rotation of proteins. This is illustrated by the data in Table VI taken from the work of Pauli and Weiss (48) on the influence of saturated solutions of urea and sodium salicylate.

1	AB	LE	V.	[
(a)	Ov	alb	un	iin

Medium H_2O Na salicylate, saturated Urea saturated Specific rotation -37° -38° -89°

(b) Serum Albumin

Medium H_2O Na salicylate, saturated Urea saturated Specific rotation -54° -72° -114°

(Pauli, W., and Weiss, R., Biochem. Z., 233, 381 (1931).)

5. ROTATORY DISPERSION

In general, the optical rotation of a compound varies inversely as the wave length of the light used for the determination. A satisfactory mathematical explanation for this phenomenon was developed in 1907 by Drude, which, in its simplest form, is embodied in the equation,

 $(\alpha)_{\lambda} = \frac{k}{\lambda^2 - \lambda_0^2}$

where $(\alpha)_{\lambda}$ is the specific rotation of the wave length of an oscillating electron in the molecule which, as a consequence, gives rise to an absorption band in the spectrum of the dissolved substance. From this it is apparent that a direct relationship exists between the rotatory dispersion and the absorption spectrum of the substance. Depending on the nature of the absorption spectrum, the rotation in the simplest case is controlled by a one term Drude equation, but may, in more complicated cases, require an equation with more terms.

Lowry (49) has shown that if the rotatory powers of a compound can be expressed by a single term Drude equation, a linear relationship is obtained on plotting the reciprocals of the rotation values, $1/(\alpha)_{\lambda}$, against the square of the wave lengths, λ^2 . This is apparent if the Drude equation is rearranged into the form,

$$\frac{1}{(\alpha)_{\lambda}} = \frac{\lambda^2}{k} - \frac{\lambda_0^2}{k}$$

It also follows from the rearranged equation that, at the point where $1/(\alpha)_{\lambda}=0$, the value of λ becomes λ_0 . In a plot of this sort, the point at which the straight line cuts the zero axis will indicate the wave length of the absorption band which controls the dispersion.

Work on the rotatory dispersion of proteins has been carried out by Hewitt (50), Jessen-Hansen (38), Wiles and Gortner (51), and

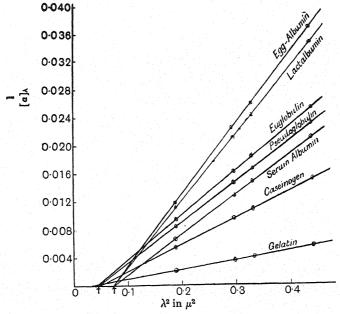


Fig. 24. Dispersion curves of proteins. Relation between $1/[a]_{\lambda}$ and λ^2 . (Hewitt, L. F., Biochem. J., 21, 216 (1927).)

Carpenter and Lovelace (47). The rotatory dispersion curves obtained by Hewitt for ovalbumin, lactalbumin, the serum proteins, casein, and gelatin, plotted in the form of the reciprocal of the rotation against the square of the wave lengths, are reproduced in Fig. 24. Since the points fall well on a straight line, the dispersion of these proteins appears to be determined by a single term Drude equation.

Since the work of Carpenter and Lovelace on gelatin is the most complete study of the optical dispersion of a protein, extensive consideration of this topic will be confined to the results given in their communication.

Carpenter and Lovelace investigated the rotatory dispersion of gelatin in sodium iodide solution primarily for the purpose of ascertaining the cause of the rapid decrease in rotation experienced with iodide salts at low temperatures. The specific rotations of gelatin solutions at the temperatures of 0.5° and 40° were measured at five different wave lengths in the visible spectrum, namely, the red lithium line at 6708 Å, the sodium line at 5893Å, the yellow

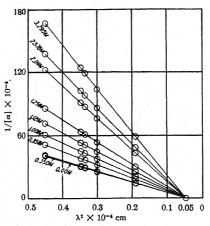


Fig. 25. Graph of reciprocal of specific rotation versus wave length squared at (0.5°).

(Carpenter, D. C., and Lovelace, F. E., J. Amer. Chem. Soc., 57, 2342 (1935).)

mercury line at 5780 Å, the green mercury line at 5461 Å, and the deep blue mercury line at 4358 Å. On plotting the reciprocal of the specific rotation values at each concentration of sodium iodide against the square of the wave length, linear relationships were obtained at both temperatures. The graph at 0.5° is shown in Fig 25. Since the relationship is linear, a single-term Drude equation adequately represents the dispersion of gelatin, as is also true for the other proteins which have so far been investigated.

The straight lines of the plots at the two temperatures of all of the sodium iodide levels cut the λ^2 axis at the same point, thus locating the value of λ_0 at the wave length of 2200 Å. Gelatin is known to have a tremendous absorption of ultraviolet light in the neighborhood of 2200 Å.

On plotting the values of the dispersion constants against the concentrations of sodium iodide, the curves reproduced in Fig. 26 are obtained. In the figure the upper curve represents the data at 0.5°, and the lower one those at 40°. At 40° the dispersion constant values bear a linear relationship to the concentration of sodium iodide, which may be expressed by the equation,

At 0.5°, the dispersion curve at varying salt concentration is made up of two parallel straight lines at the extreme ends, united by a curve of the form representing a dissociation or association process.

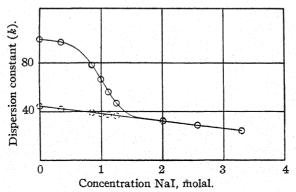


Fig. 26. Rotatory dispersion constant of gelatin in sodium iodide solutions at 0.5 and 40° .

(Carpenter, D. C., and Lovelace, F. E., J. Amer. Chem. Soc., 57, 2342 (1935).)

The rapidly changing part of the curve may be represented by the equation

 $[\text{NaI}] = \frac{1}{2.66} \log \frac{\alpha}{1 - \alpha} + \log 1/k$

where α represents the dissociated fraction as shown by the change in magnitude of the dispersion constants, $k_{0.5}$ °.

The $\log 1/k$ value is specifically representative of sodium iodide. Different values of $\log 1/k$ are obtained for the other halides. These constants have been approximately calculated as 1.0 for the iodide, 1.8 for the bromide, and 3.7 for the chloride ion. The factor 1/2.66 preceding the dissociation term is indicative of the rapidity of the dissociation.

From this evidence the authors conclude that the transformation embodied in the change from "gel" to "sol" state is a process of either a dissociation or association of the gelatin molecule.

The authors have also applied the determinant method of Lucas (52) to estimate the number of optically active species in the gelatin system. The analysis yielded results which indicated that only two optically active components are present. This test is offered as a confirmation of their theory of the sol-gel transformation of gelatin.

It has been pointed out by L. Pauling (*) that, if the optical ro-

^{*} Personal communication.

tation changes depend upon an equilibrium between a state of association and dissociation, then a change of concentration in gelatin should produce the same effect as the influence of temperature or the alkali halides on the rotation. Since this has not been shown to occur, the more likely explanation is that two tautomeric forms of gelatin with different optical properties exist, and that the sol-gel transformation is associated with mutarotation in the gelatin molecule. This viewpoint harmonizes with the theory of the optical change proposed by Smith in 1921.

The deductions drawn from the measurements of the rotatory dispersion of gelatin in solutions of sodium iodide have been confirmed by Carpenter and Lovelace (53) with sodium bromide.

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PART II. CHEMICAL DYNAMICS OF AMINO ACIDS AND PROTEINS

CHAPTER XI

AMPHOTERIC PROPERTIES OF AMINO ACIDS AND PROTEINS

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1. AMPHOTERIC PROPERTIES OF AMINO ACIDS

An amphoteric electrolyte or ampholyte is a substance which exhibits both acidic and basic properties. In terms of the classical ionic theory such a substance, when dissolved in water, may form both hydrogen ions and hydroxyl ions, as well as anions and cations consisting of the rest of the ampholyte molecule.

The first amino acids were discovered early in the nineteenth century, long before the formulation of the ionic theory, but their amphoteric nature was at once recognized, as has been pointed out in a recent review (1). Compounds of amino acids with acids such as hydrochloric acid and with bases such as sodium hydroxide were soon isolated, and their formation was accounted for by the basic properties of the amino group and the acidic properties of the carboxyl group. The absence of a strongly acid or alkaline reaction in aqueous solutions of glycine was explained by Strecker (2) by the assumption that the acidic group of glycine could combine with the basic part of the same molecule. This idea of inner salt formation was developed by Bredig (3) into the concept of the formation of Zwitterionen (amphions or dipolar ions); such an ion is assumed to have at the same time both a positive and a negative charge, located on different groups.

The quantitative characterization of the amphoteric properties of an amino acid involves a knowledge of the dissociation constants which describe its acidic and basic ionization. Such knowledge is obtained by applying the law of mass action to the results of measurements which have been made with solutions of compounds of amino acids.

(1) Classical Formulation of Dissociation Equations. The two

types of ionization of amino acids were first described by equations of the form

 $NH_2RCOOH \rightleftharpoons NH_2RCOO^- + H^+$, or $R \rightleftharpoons R^- + H^+$ and $HONH_3RCOOH \rightleftharpoons +NH_3RCOOH +OH^-$, or $R \rightleftharpoons R^+ +OH^-$

The corresponding mass law equations are

$$k_a = \frac{a_{\rm H} + a_{\rm R}}{a_{\rm R}}$$
 and $k_b = \frac{a_{\rm R} + a_{\rm OH}}{a_{\rm R}}$

where the symbol a represents activity as defined by Lewis (4). (Here no distinction is made between the hydrated and unhydrated forms of the uncharged molecule, since the amount of each is a constant fraction of their sum, which is all that has been determined by experiment.) Such equations were applied on the assumption that k_a would be given by measurements with alkaline solutions of the ampholyte, and k_b by those with acid solutions. Values of these constants for several amino acids were obtained by Winkelblech (5) from measurements of the conductivity of solutions of their salts with a strong acid or base. The values so obtained were very small, being about 10^{-9} to 10^{-10} for k_a and 10^{-11} to 10^{-12} for k_b . Since the amino acids may be regarded as substituted derivatives of acetic acid and ammonium hydroxide, which have ionization constants about equal to 1.8×10^{-5} , an acceptance of these low figures for the amino acids implies a tremendous influence of the substituent groups on electrolytic dissociation.

(2) Zwitterion Formulation of Dissociation Equations. It was noticed by Adams (6) and by Bjerrum (7) that much more reasonable values for the constants of amino acids could be obtained from the same experimental data by assuming the uncharged fraction of the amino acid to exist mostly in the form of doubly-charged Zwitterionen or dipolar ions. On this basis the ionization relations are described by the equilibria,

$$+NH_3RCOOH \rightleftharpoons +NH_3RCOO^- + H^+ \text{ or } R^+ \rightleftharpoons R^\pm + H^+$$

 $+NH_3RCOO^- \rightleftharpoons +NH_3RCOO^- + OH^- \text{ or } R^- \rightleftharpoons R^\pm + OH^-$

for which the mass law expressions are:

and

$$K_A = \frac{a_{\rm H}^+ a_{\rm R}^{\pm}}{a_{\rm R}^+}$$
 and $K_B = \frac{a_{\rm R}^{\pm} a_{\rm OH}^-}{a_{\rm R}^-}$

This differs from the classical formulation in two respects. First,

the uncharged molecule is written as an amphion. Second, the acidic ionization of the carboxyl group takes place during the neutralization of an acid solution, while the basic ionization is favored by the neutralization of an alkaline solution which contains the ampholyte anion. Evidently measurements which yield values for k_a of the classical theory will give values for K_B of the amphion theory and measurements giving k_b will also give K_A . The two sets of constants may be related by identifying a_R and a_R , since each refers here to the total electrically neutral ampholyte. If the equations defining K_A and k_b are multiplied together, as are those defining K_B and k_a , it follows that

$$K_A k_b = a_{\mathbf{H}} + a_{\mathbf{OH}} - K_B k_a$$

These products are equal to the ion product for water, since

$$K_w = a_{\rm H} + a_{\rm OH} = [H^+][OH^-]\gamma_{\rm H} + \gamma_{\rm OH}$$
 (1)*

for any solution so dilute that the activity of unionized water may be taken as unity. The desired relations are, therefore,

$$K_A = \frac{K_w}{k_b}$$
 and $K_B = \frac{K_w}{k_a}$

It is to be emphasized that there is no doubt as to the formulas for the cation and anion of any amino acid, which are the same in both systems, and that the experimental results may be described in terms of either system of notation with equal exactness. The advantage of the *Zwitterion* system is that it gives more reasonable values to the constants, which are, approximately, $K_A = 10^{-2}$ to 10^{-3} and $K_B = 10^{-4}$ to 10^{-5} .

(3) Formulation of Dissociation Equations in Terms of Acidic Ionization. On the basis of Brönsted's definition of acids and bases (8), all ionization constants may be written as acid constants. Instead of writing

$$\mathrm{NH_4OH} \rightleftarrows \mathrm{NH_4^+} + \mathrm{OH^-}; \ k_b = \frac{a_{\mathrm{NH_4}^+} a_{\mathrm{OH}^-}}{a_{\mathrm{NH_4OH}}}$$

* In this and other mass law equations a refers to the activity, the brackets to the molal concentration, and γ to the activity coefficient of the ion or molecule. These quantities, as defined by Lewis (4), are connected by the relation

 $a_x = [X]\gamma_x$ where X indicates the molecular or ionic species in question. At very low concentrations all activity coefficients approach unity and the activity tends to become identical with the molality.

Brönsted writes

$$NH_4^+ \rightleftharpoons NH_3 + H^+, K_A = \frac{a_H^+ a_{NH_3}}{a_{NH_4^+}}$$

Evidently the two constants describe the same reaction, and are simply related through the ionization of water:

$$K_A = \frac{K_w}{k_b}$$

Thus any base is related to its conjugate acid, and its ionization may be formulated in terms of the tendency of the conjugate acid (in this case, NH₄+) to lose hydrogen ions or protons (which are probably hydrated in aqueous solutions). Brönsted's system has the advantage of being applicable to non-aqueous solutions in which hydroxyl ions may not appear.

The application of this system to the ionization of an amino acid is made by considering the ampholyte cation as a dibasic acid:

$$+NH_3RCOOH \rightleftharpoons +NH_3RCOO-+H^+$$
, or $R^+ \rightleftharpoons R^{\pm}+H^+$
 $+NH_3RCOO- \rightleftharpoons NH_2RCOO-+H^+$, or $R^{\pm} \rightleftharpoons R^-+H^+$

$$K_{1} = \frac{a_{\mathrm{H}} + a_{\mathrm{R}} \pm}{a_{\mathrm{R}} + \frac{[\mathrm{H}^{+}][\mathrm{R}^{\pm}] \gamma_{\mathrm{H}} + \gamma_{\mathrm{R}} \pm}{[\mathrm{R}^{+}] \gamma_{\mathrm{R}} + }}$$
(2)

$$K_2 = \frac{a_{\rm H}^+ a_{\rm R}^-}{a_{\rm R}^{\pm}} = \frac{[{\rm H}^+][{\rm R}^-] \gamma_{\rm H}^+ \gamma_{\rm R}^-}{[{\rm R}^{\pm}] \gamma_{\rm R}^{\pm}}$$
(3)

Here the electrically neutral form has been written as an amphion to indicate that the ionization of the carboxyl group probably occurs when an acid solution containing the cation is partly neutralized by alkali, while the removal of hydrogen ion from the substituted ammonium group probably occurs when alkali is added to the electrically neutral form. It is still true, however, that the experiments by which the constants are determined do not give direct information as to which constant refers to the carboxyl group and which to the amino group. Again the experiments are accurately described by either system. Evidently the acidic constants are related to those of the other systems as follows:

$$K_1 = K_A = \frac{K_w}{k_b}$$

$$K_2 = \frac{K_w}{K_R} = k_a$$

This system of acidic constants has the advantage that the values of K_1 and K_2 are directly related to those of $a_{\rm H}^+$ in the solutions used in the experiments by means of which the values of the constants are determined. This is desirable because most of the accepted values for these constants have been obtained from electrometric measurements involving $a_{\rm H}^+$. In the remainder of this chapter, all ionization constants of amino acids will be given as constants of acidic ionization, in agreement with the notation of recent workers (9, 10).

(4) The Isoelectric Condition. When a solution of an amphoteric substance is placed between electrodes which are connected to a source of direct current, the particles or ions of the ampholyte will, in general, migrate towards one pole or the other. This phenomenon was studied by Hardy (11), who worked with solutions, previously boiled, containing the protein material of egg white. These particles moved towards the negative pole in acid solutions and towards the positive pole in alkaline solutions. At some intermediate degree of acidity the particles showed no migration towards either pole; they were then said to be isoelectric. Michaelis (12) combined such migration experiments with hydrogen electrode measurements of pH, and found that for each amphoteric substance there was a definite pH value, or zone of pH values, corresponding to the absence of migration. Such migration of an ampholyte is affected more by changes in acidity than by other conditions; accordingly, it has become customary to define isoelectric points in terms of the pH scale. The concept of the isoelectric condition, however, is not fundamentally connected with the pH scale, for it could be defined in terms of any complete description of the solution in which no migration takes place.

Evidently a simple amino acid will be in the isoelectric condition when the concentrations of its cation and anion are equal. This condition may be related to the concentration of hydrogen ions in the solution. If equations (2) and (3) are multiplied together, it follows that, for any solution of the ampholyte,

$$K_1 K_2 = \frac{a_{\rm H}^{+2} a_{\rm R}^-}{a_{\rm R}^+} = \frac{[{\rm H}^+]^2 [{\rm R}^-] \gamma_{\rm H}^{+2} \gamma_{\rm R}^-}{[{\rm R}^+] \gamma_{\rm R}^+}$$

At the isoelectric point $[R^+]$ and $[R^-]$ are equal. Hence, if I is the value of $[H^+]$ at the isoelectric point,

$$I^{2} = K_{1}K_{2} \frac{\gamma_{R}^{+}}{\gamma_{R} \gamma_{R}^{+2}}$$
 (4)

Michaelis (12) showed that a similar equation could be obtained by defining the isoelectric point, I, for this simple case, as the value of $[H^+]$ at which the electrically neutral (uncharged or Zwitterion) fraction of the ampholyte is at a maximum.

The quantity pH is now usually defined as equal to $-\log a_{\rm H}^+$, or $-\log [{\rm H}^+]\gamma_{\rm H}^+$ (this is Sörensen's (13) paH), and if pI' is used to represent the value of pH at an isoelectric point, we have

$$pI' = \frac{1}{2} \left(pK_1 + pK_2 - \log \frac{\gamma_R^+}{\gamma_R^-} \right)$$

where pK_1 and pK_2 are the dissociation indices, or negative logarithms of the acidic ionization constants, of the ampholyte. If the apparent pK' values, as determined from pH measurements, are used, the activity coefficient of the hydrogen ion is included in pI' and those of the ampholyte are included in the pK' values, or

$$pI' = \frac{1}{2}(pK_1' + pK_2') \tag{4a}$$

If the ampholyte has more than two ionizable groups, theoretically all of the ionization constants will be effective in determining the location of the isoelectric point (14). This may be seen from the following equation for the isoelectric point of a multivalent ampholyte in terms of all of its ionization constants (15):

$$I^{2} = K_{n}K_{n+1} \frac{1 + \frac{2K_{n+2}}{I} + \frac{3K_{n+2}K_{n+3}}{I^{2}} + \cdots}{1 + \frac{2I}{K_{n-1}} + \frac{3I^{2}}{K_{n-1}K_{n-2}} + \cdots}$$

Here the K values refer to acidic constants, numbered as usual in order of decreasing magnitude, the subscript n being equal to the highest positive valence of any of the ampholyte ions. In all known cases the value for I lies between those of K_n and K_{n+1} , so that usually I is much greater than K_{n+2} and much less than K_{n-1} . Ordinarily, therefore, if the proper activity coefficients are inserted, this equation reduces to equation (4).

The isoelectric points of many simple amino acids are not sharply defined, but may extend over a zone of several pH units. Michaelis (12) showed that a narrow zone is obtained only if the value for K_1 is less than ten thousand times that of K_2 ; that is, if pK_1 and pK_2 are less than about 4 units apart. His diagram shows

also that when the isoelectric region is narrow, a considerable amount of the ampholyte is present as cations and anions, even at the isoelectric point. This amount would be 67 per cent if K_1 and K_2 could be equal, 39 per cent if K_1 were 10 times K_2 , and 17 per cent if K_1 were 100 times K_2 (15).

A solution of a pure amino acid in water will not, in general, be exactly isoelectric. Theoretically this could be the case only if $K_1 \cdot K_2 = K_w$, and such an ampholyte would be isoelectric at the neutral point of pure water. The pH value of a solution of any other ampholyte, with no added acid or base, lies between that of its isoelectric point and that of pure water, but approaches the former as the concentration is increased. Sörensen (16) gave a formula for calculating the pH of such a solution, and showed that the pH of a 1.0 M glycine solution would differ from that of the isoelectric point by only 0.001 pH unit. He also pointed out that a pure ampholyte becomes isoelectric in a solution containing only enough of a strong acid or base to bring the pure solvent to the isoelectric pH for the ampholyte. Similarly, Michaelis (12) found it possible to determine the isoelectric point of a pure ampholyte such as glycine or phenylalanine by dissolving it in a series of dilute buffer solutions of varied pH; the addition of the ampholyte produced a change in pH in every solution except that which was initially at the pH of the isoelectric point of the ampholyte used.

Theoretically, the isoelectric condition of an ampholyte may depend on its combination, not only with hydrogen or hydroxyl ions, but also with other anions or cations which may be in the solution. Sörensen (13) has therefore defined the *isoionic point* of an ampholyte as the hydrogen ion activity at which it is combined equally with H^+ and with OH^- , and has pointed out that this is identical with the isoelectric point only if the substance does not combine with ions other than hydrogen and hydroxyl. Strictly, therefore, it is only the isoionic point which is obtained from pK values by equation (4) or (4a), or by the experiments in which no change in pH is produced. Only the method of electrical migration would give the isoelectric point in every conceivable case.

(5) Determination of Dissociation Constants. The values of K_1 and K_2 in equations (2) and (3) may be determined by any type of experiment which gives values for the concentration terms. The concentrations are measured best in solutions containing not only the amino acid but also some strong acid or base. The conductivity method, as used in early work (5), involved doubtful assumptions,

and later work in Schmidt's laboratory (to be discussed in Chapter XII) has indicated that conductivity measurements do not readily yield exact values for the dissociation constants of amino acids. Other methods which are, in general, more satisfactory for this purpose are based on the increased solubility of amino acids in solutions of a strong acid or base, on hydrogen electrode titrations (pH determinations), or on the electromotive force of cells without liquid junction.

In these three methods use is made of equation (2), or equations (1) and (3), with certain additional relations between total and ionic concentrations. For solutions containing an ampholyte at the total concentration C and a strong acid at the total concentration A, the equations

$$A = [H^{+}] + [R^{+}] \tag{5}$$

$$C = [R^{\pm}] + [R^{+}] \tag{6}$$

must hold. For solutions containing the ampholyte at the total concentration C and a strong base at the total concentration B, the corresponding relations,

$$B = [OH^{-}] + [R^{-}]$$
 (7)

$$C = [R^{\pm}] + [R^{-}] \tag{8}$$

are evident. Neglecting for the moment the activity coefficients, the three equations (2), (5), and (6) contain four unknown quantities, while the four equations (1), (3), (7), and (8) contain five unknown quantities. Each of the methods of obtaining K_1 or K_2 must therefore provide an additional equation containing one of the same unknown quantities, as well as some means of estimating or eliminating the activity coefficients.

(6) Dissociation Constants from Solubility Measurements. Here the additional relation is given by the principle that the activity of the saturating body in a saturated solution, at constant temperature, is independent of the composition of the solution. If the solubility of a slightly soluble ampholyte is measured in pure water (strictly, at its isoelectric point) and in hydrochloric acid or in sodium hydroxide solutions, we have the relation,

$$a_{\mathbf{R}^{\pm}} = [\mathbf{R}^{\pm}] \gamma_{\mathbf{R}^{\pm}} = C_0 \gamma_0 \tag{9}$$

in which C_0 and γ_0 are the concentration and the activity coefficient of the ampholyte in the isoelectric condition. For an ampholyte having a broad isoelectric zone, C_0 is identical with the total solu-

bility in pure water. For the solutions in hydrochloric acid, equations (2),(5),(6) and (9) may be combined to give

$$K_{1} = \left(\frac{A}{C - C_{0} \frac{\gamma_{0}}{\gamma_{R}^{\pm}}} - 1\right) \cdot C_{0} \cdot \frac{\gamma_{0} \gamma_{H}^{+}}{\gamma_{R}^{+}}$$

$$(10)$$

An apparent dissociation constant K_{1} ' may be defined by the equation

 $K_1' = \left(\frac{A}{C - C_0} - 1\right) \cdot C_0 \tag{11}$

and K_1 when extrapolated to zero ionic strength (4) must become identical with K_1 . The writer has attempted such an extrapolation with his data (17) for the solubility of tyrosine in hydrochloric acid, and found no significant variation in $\log K_1$ with the ionic strength. This is taken to mean that for this case the two ratios of activity coefficients in equation (10) are close to unity. Evidently this method will give more accurate values for K_1 when the increase in solubility, $C - C_0$, is large; that is, when the acid concentration, A, is not too small.

For the case of a simple amino acid in sodium hydroxide solutions, equations (1), (3), (7), (8), and (9) give the relation,

$$\frac{K_w}{K_2} = \left(\frac{B}{C - C_0 \frac{\gamma_0}{\gamma_{\text{R}^{\pm}}}} - 1\right) \cdot C_0 \cdot \frac{\gamma_0 \gamma_{\text{OH}^-}}{\gamma_{\text{R}^-}} \tag{12}$$

An apparent dissociation constant, K_2 , may be defined by

$$\frac{K_w}{K_2'} = \left(\frac{B}{C - C_0} - 1\right) \cdot C_0 \tag{13}$$

and log K_2 ' should be a linear function of the ionic strength, giving a value for log K_2 by extrapolation to zero ionic strength. No data have been found by which this method may be tested. The case of tyrosine is complicated by the dissociation of the hydroxyl group; it was necessary to use pH measurements in order to obtain an estimate of the two overlapping constants which are effective in determining its solubility in alkaline solutions (17).

(7) Dissociation Constants from pH Measurements. Here the additional equation is the definition of pH,

$$pH = -\log \alpha_{H^{+}} = -\log [H^{+}]\gamma +$$
 (14)

Solutions are prepared containing the ampholyte at a constant concentration, C, and also a strong acid or base at varied concentrations, A or B. In the former case the pH values are used in equation (2), which is now written best in logarithmic form, together with equations (5) and (6), giving

$$pK_1 = pH - \log\left(\frac{C}{A - [H^+]} - 1\right) - \log\frac{\gamma_R^{\pm}}{\gamma_R^{+}}$$
 (15)

$$pK_1' = pH - \log\left(\frac{C}{A - [H^+]} - 1\right)$$
 (16)

In order to use these equations, [H+] must be calculated from the pH measurements by assuming values for $\gamma_{\rm H}$ ⁺. The best assumption is that $\gamma_{\rm H}$ ⁺ has the same value as in solutions of the same ionic strength containing the strong acid in water alone.

For solutions in alkali, equations (3), (7), (8) and (14) give

$$pK_2 = pH + \log\left(\frac{C}{B - [OH^-]} - 1\right) - \log\frac{\gamma_{R^-}}{\gamma_{R^{\pm}}}$$
 (17)

$$pK_{2}' = pH + \log\left(\frac{C}{B - [OH^{-}]} - 1\right)$$
 (18)

Values of [OH-] are obtained from equation (1) by using the pH data, with the assumption that $\gamma_{\rm OH}$ - has the same values as in pure sodium hydroxide solutions of the same ionic strength. It is desirable to use values of $K_w/\gamma_{\rm OH}$ - based on electrometric pH determinations, made under the same conditions, using pure sodium hydroxide solutions.

This method has yielded almost all of the results which are now available for the dissociation constants of amino acids. These results have usually been reported as pK' rather than pK, since values which were obtained at different ionic strengths have been averaged. A notable exception is the work of Bjerrum and Unmack (10), who obtained, by extrapolation, true values of pK_1 and pK_2 for glycine. Their data indicate that pK_1' is about 0.04 to 0.08 greater than pK_1 at ionic strengths of 0.01 to 0.10, while pK_2' is less than pK_2 by about the same amounts.

This method of obtaining the pK' values for a simple amino acid may be illustrated by the following recalculations of some of the data of Sörensen (18, 16) on the pH values of glycine buffer solutions. These data were first published in 1909, in the classical paper

in which the symbol pH was first proposed, and Sörensen did not attempt to use them to calculate dissociation constants but merely as a means of standardizing the colorimetric determination of pH. Each solution was prepared by mixing a stock solution, 0.1 M with respect to glycine and sodium chloride, with 0.1 M hydrochloric acid or sodium hydroxide. The ionic strength of all of the solutions was therefore 0.1 M. The results are shown in Table I.

Table I

Calculation of pK₁' and pK₂' for Glycine at 18° from pH Measurements

(Data of Sörensen, S. P. L., Biochem. Z., 21, 131 (1909).)

Glycine+HCl+NaCl, ionic strength 0.1 M.

A = HCl per liter	C = gly- cine per liter	pН	-log[H ⁺]	$\frac{A - [H^+]}{C}$ = fraction of glycine as cation	$\log\left(\frac{C}{A - [H^+]} - 1\right)$	pK_1'
moles 0.100 0.050 0.040 0.030 0.020 0.010 0.005	moles 0 0.050 0.060 0.070 0.080 0.090 0.095	1.038 1.932 2.279 2.607 2.922 3.341 3.679	1.000 1.894 2.241 2.569 2.884 3.303 3.641	0.745 0.571 0.390 0.234 0.1056 0.0502	-0.466 -0.124 $+0.194$ 0.515 0.928 1.277	2.398 2.403 2.403 2.407 2.413 2.402
					Average	2.404

Glycine + NaOH + NaCl, ionic strength 0.1 M

B = NaOH per liter	$C = \operatorname{gly-}$ cine per liter	рН	-log [OH-]	$\frac{B - [OH^-]}{C}$ = fraction of glycine as anion	$\log \left(\frac{C}{B - [\text{OH}^-]} - 1 \right)$	pK_2'
moles	moles					
0.100	0	13.066	1.000			
0.050	0.050	11.305	2.761	0.965	-1.441	9.864
0.040	0.060	10.140	3.926	0.665	-0.297	9.843
0.030	0.070	9.714	4.352	0.428	+0.126	9.840
0.020	0.080	9.364	4.702	0.250	0.477	9.841
0.010	0.090	8.929	5.137	0.1110	0.904	9.833
0.005	0.095	8.575	5.491	0.0526	1.255	9.830
		11/1			Average	9.842

In the first part of the table the values of $[H^+]$ were obtained from equation (14), the modern definition of pH, by assuming that $-\log \gamma_{\rm H^+}$ was 0.038, a figure obtained from the pH value then assigned to 0.1 M hydrochloric acid. In the second part of the table the values of $-\log [{\rm OH^-}]$ were obtained by subtracting the pH values from 14.066; this figure was obtained by the use of the same definition of pH and the ion product law for water, equation (1), which may be written

$$-\log [OH^-] = -\log K_w + \log \gamma_{OH^-} - pH$$

From the data for 0.1 M sodium hydroxide:

$$-\log K_w + \log \gamma_{\text{OH}} - \text{pH} - \log [\text{OH}^-] = 13.066 + 1.000 = 14.066$$

and this figure must apply to all of the solutions if the ionic strength principle (4) is valid.

The constancy of the pK' values in Table I shows that the ionization of glycine is very well described by the law of mass action, and testifies, at the same time, to the remarkable accuracy of these early measurements of Sörensen. The values of pK' may be subject to slight revision because Sörensen's pH value for 0.1 M hydrochloric acid is about 0.04 lower than the current value (1.075) and also because he used the Bjerrum extrapolation for the estimation of liquid junction potentials. The latter were estimated in a different way by Bjerrum and Unmack (10), who studied glycine twenty years after Sörensen's work appeared. Their pK' values for 18° and an ionic strength of 0.1 M are 2.43 and 9.88. If the average values of Table I are increased by 0.04, to reconcile the difference in the pH scales, they become 2.44 and 9.88.

The results of such studies with the hydrogen electrode have often been presented in the form of titration curves, by plotting the values of A or B, with C constant, against pH. A corrected titration curve, which is also the combination or dissociation curve of the ampholyte, is obtained by plotting $[A-[H^+]]/C$ and $[B-[OH^-]]/C$ against pH. The dissociation curve of glycine is shown in Fig. 1, which was constructed from the data in Table I. Similar curves have been obtained for other simple amino acids, which have been studied extensively by Schmidt and his collaborators (19). Each section of these corrected curves is similar in shape to the theoretical dissociation curves described by Michaelis (12) and by Clark (20), which are also plots of the ratios, $[A-[H^+]]/C$ or $[B-[OH^-]]/C$, against pH. Equations (16) and (18) show that

pH will be equal to pK' when one of these ratios has the value 0.5; this occurs in the middle of the region of maximum buffer value, and the best values of pK' are obtained when the ratio C:A or C:B is not far from 2:1.

If any two pK values for the same substance are less than about three units apart, the steps in the corrected titration curve, shown in Fig. 1, may not be evident. In such cases the constants are said to overlap, and their exact determination is a matter of some difficulty. The necessary calculations have been given by Simms (21),

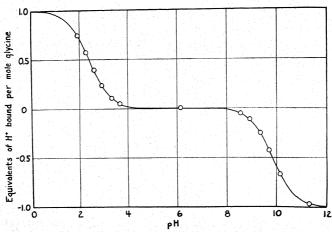


Fig. 1. Dissociation curve of glycine at 18° and ionic strength 0.1 M. The points are experimental, recalculated by the writer from Sörensen's pH measurements. The curve is theoretical, based on the constants in Table I.

(Sörensen, S. P. L., Biochem. Z., 21, 174 (1909).)

who showed that the titration curve of any multivalent weak electrolyte is identical with that of a mixture of univalent weak acids having suitable dissociation constants, G'. These constants, G', are called the titration constants of the multivalent substances. Simms showed how to obtain their values from a single titration curve by a method involving successive approximations. The titration constants, G', are equal to the apparent dissociation constants, K', of the multivalent substance only when the constants do not overlap; that is, when successive pK' values are not less than 2.5 or 3.0 units apart. Exact equations relating the G' and K' values for all cases were given by Simms (21).

Dissociation curves of ampholytes with several ionizable groups have been obtained by Simms (22) and by Greenstein (23), who used this method of calculation (21). In Figs. 2 and 3 (23) the open

circles refer to the dissociation data of histidine and of aspartic acid. The former substance contains two basic groups and one carboxyl group; the latter, one amino group and two carboxyl groups. In each case there is overlapping of the segments of the curves at the isoelectric point, which is at pH 7.6 for histidine and at pH 3.0 for aspartic acid. (The other curves in Figs. 2 and 3 will be discussed later.)

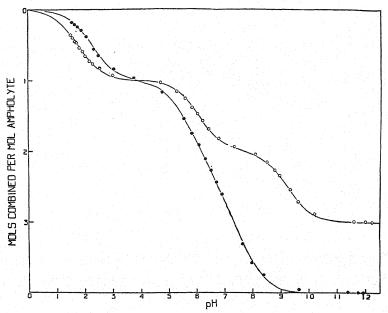


Fig. 2. Dissociation curves of histidine (open circles) and histidyl-histidine (inked circles). The points are experimental. The curves are theoretical, based on the constants in Table II. The ordinates are equivalents of H⁺ removed from 1 mole of the cation of the ampholyte.

(Greenstein, J. P., J. Biol. Chem., 93, 479 (1931).)

(8) Dissociation Constants from Cells Without Liquid Junction. A more accurate method for the determination of the dissociation constants of weak electrolytes is that of Harned and his co-workers (24), who used the electromotive force of cells without liquid junction in such a way that the constants could be obtained with practically no extra-thermodynamic assumptions. This method has been successfully applied to the amino acids alanine (25) and glycine (26).

¹ Thermodynamic constants for seven additional amino acids have recently been obtained in the writer's laboratory (90).

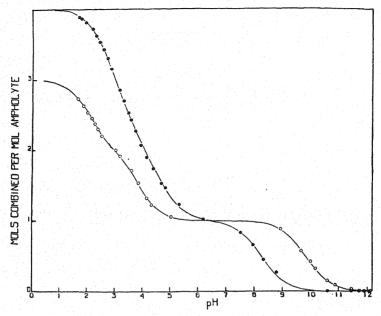


Fig. 3. Dissociation curves of aspartic acid (open circles) and aspartyl-aspartic acid (inked circles). The points are experimental. The curves are theoretical, based on the constants in Table II. The ordinates are equivalents of H⁺ bound by 1 mole of the anion of the ampholyte.

(Greenstein, J. P., J. Biol. Chem., 93, 479 (1931).)

The cells which were used are:

and

The electromotive force of either cell is given by the exact thermodynamic equation,

$$E = E_0 - k \log [H^+][Cl^-]\gamma_{H^+}\gamma_{Cl^-}$$
 (19)

in which k is 2.3026 RT/F and E_0 is a constant dependent only on the temperature, its values being accurately known.

For an amino acid in hydrochloric acid solutions, equations (2), (5), (6), and (19) may be combined to give

$$pK_1 + \log \frac{\gamma_{\mathrm{H}}^{+}\gamma_{\mathrm{R}}^{\pm}}{\gamma_{\mathrm{R}}^{+}} = \frac{E - E_0}{k} + \log A - \log \left(\frac{C}{A - [\mathrm{H}^{+}]} - 1\right) + \log \gamma_{\mathrm{H}}^{+}\gamma_{\mathrm{Cl}}^{-}$$

$$(20)$$

All quantities in the right member are known except [H+] and the product of the activity coefficients. For purposes of extrapolation,

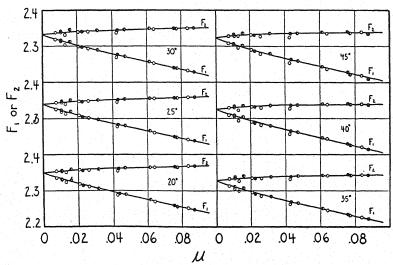


Fig. 4. Electrometric determination of pK_1 for alanine by a cell without liquid junction. The abscissas are the ionic strength values of the solutions; the ordinates are the values of the right member of equation (20), calculated by two assumptions as to activity coefficients. The intercept at zero ionic strength is the value of pK_1 . (Nims, L. F., and Smith, P. K., J. Biol. Chem., 101, 401 (1933).)

the values of the latter may be assumed to be the same as for pure hydrochloric acid of the same ionic strength, or they may be calculated from the theoretical limiting law of Debye and Hückel. Values of [H+] are obtained by using these activity coefficients, with the known value of [Cl-] (which is A in this case), in equation (19). The values of the right member of equation (20), when plotted against the ionic strength, fall on a line which is nearly straight. This makes it possible to obtain the true constant pK_1 by extrapolation to zero ionic strength, where the logarithm in the left member becomes zero. Both assumptions as to the activity coefficients yield the same extrapolated value for pK_1 , which is believed to be known within ± 0.003 units. The nature of the extrapolation is illustrated by Fig. 4 (25), in which F_2 and F_1 are the values obtained for the right member of equation (20) by the two assumptions mentioned.

For a solution containing an amino acid of concentration C, sodium hydroxide of concentration B, and sodium chloride of concentration [Cl⁻], equations (3), (7), (8), and (19) are combined to give

$$pK_2 - \log \frac{\gamma_{\text{Cl}} \gamma_{\text{R}^{\pm}}}{\gamma_{\text{R}^{-}}} = \frac{E - E_0}{k} + \log \left[\text{Cl}^- \right] + \log \left(\frac{C}{B - [\text{OH}^-]} - 1 \right)$$
 (21)

Here all of the quantities on the right are known except [OH-], and its value may be obtained with sufficient accuracy by the use of equations (1) and (19), with the assumption that γ_{OH}^- and γ_{CI}^- are identical for the same solution. Then the values of the right member of equation (21) are plotted against the ionic strength and the extrapolation is carried out as before. Equation (21), like equation (20), has been written in such a way as to make the value of each member approach that of the true pK in a nearly linear way

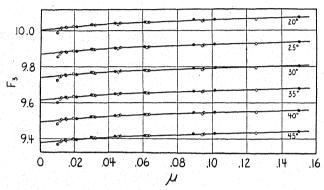


Fig. 5. Electrometric determination of pK_2 for alanine by a cell without liquid junction. The abscissas are the ionic strength values of the solutions; the ordinates are the values of the right member of equation (21). The intercept at zero ionic strength is the value of pK_2 .

(Nims, L. F., and Smith, P. K., J. Biol. Chem., 101, 401 (1933).)

as the ionic strength approaches zero. Fig. 5 shows the nature of this extrapolation for the case of alanine in sodium hydroxide solutions (25). The ordinate F_3 is the value of the right member of equation (21).

(9) Values of the Dissociation Constants of Amino Acids. In Table II are given values for the negative logarithms of the ionization constants and isoelectric points for those amino acids which have been shown to be products of the hydrolysis of proteins. More extensive tables are given by Cohn (9) and by Schmidt and co-workers (19). The table shows that almost every amino acid has one pK between 2.0 and 2.5, and another between 9 and 10. The simple amino acids have isoelectric points near pH 6, while those containing an additional amino or carboxyl group are isoelectric at more alkaline or more acid reactions. While on the classical theory the isoelectric point of a simple amino acid corresponds to minimum dissociation, on the amphion theory it corresponds to maximal charge, since the amphion is bivalent while the anion and

Table II

Dissociation Indices and Isoelectric Points of Amino Acids and Peptides at 25°

Substance	pK', and probable determining group						Ref.
	-соон	-ОН	-SH	= NH	-NH ₂	1-1-1	
Alanine $(pK \text{ at inf. dil.})$	2.340				9.870	6.1	(25)
Arginine	2.02				9.04		
					12.48*	10.8	(19)
Aspartic acid	2.09			4,111			
	3.87				9.82	3.0	(23)
Cysteine (30°)	1.96		8.18		10.28	5.1	(27)
Cystine (30°)	<1.0				7.48		
생생님 집에 지어 하다	1.7				9.02	5.6	(27)
Diiodotyrosine	2.12	6.48			7.82	4.3	(19)
Glutamic acid	2.19						
	4.28				9.66	3.2	(19)
Glycine $(pK \text{ at inf. dil.})$					9.778	6.1	(26)
Histidine	1.77			6.10	9.18	7.6	(23)
β-Hydroxyglutamic acid							
	4.24				9.56	3.3	(19)
Hydroxyproline	1.92			9.73		5.8	(19)
Isoleucine**	2.36				9.68	6.0	(19)
Leucine**	2.36	3 B			9.60	6.0	(28)
Lysine	2.18				8.95		44.0
					10.53	9.7	(19)
Methionine	2.28				9.21	5.8	(19)
Norleucine**	2.39				9.76	6.1	(19)
Phenylalanine	2.58				9.24	5.9	(19)
Proline	2.00			10.60		6.4	(19)
Serine	2.21				9.15	5.7	(19)
Tryptophane	2.38				9.39	5.9	(19)
Tyrosine	2.20	10.1			9.1	5.7	(17)
Valine**	2.32				9.62	6.0	(19)
Aspartyl-aspartic acid	2.62						
	3.46						
	4.72				8.26	3.0	(23)
Alanyl-alanine	3.17	l tan i			8.42	5.8	(22)
Glycyl-alanine	3.15				8.25	5.7	(22)
Glycyl-glycine	3.12				8.07	5.6	(22)
Glycyl-glycyl-glycine	3.26	100			7.91	5.6	(22)
Glycyl-alanyl-alanyl-		100					
glycine	3.30				7.9	5.6	(22)
Histidyl-histidine	2.25			5.60			
				6.80	7.80	7.3	(23)

^{*} The value 12.48 is determined by the guanidine group in arginine, and might be allocated to either the $-\mathrm{NH_2}$ or the $=\mathrm{NH}$ radical in this group. In this connection see Jukes, T. H., and Branch, G. E. K., Science, 80, 228 (1934).

⁽See also Cohn, E. J., Ergeb. Physiol., 33, 781 (1931).)

** The following values for the thermodynamic constants, pK_1 and pK_2 at infinite dilution and 25°, have been obtained (90): isoleucine, 2.318 and 9.758; leucine, 2.328 and 9.744; norleucine, 2.335 and 9.834; valine, 2.286 and 9.719.

cation are univalent. Cohn (9) has pointed out that on the basis of the amphion theory the diamino and dicarboxylic acids have maximal charges, not at their isoelectric points, but when the univalent cation or anion, respectively, is present in maximal amount. This must be the case if each of these ions has three charges, as R^{±+}and R^{±-}. For acids of the type R(NH₂)₂COOH the pH of maximal charge is given approximately by

$$pM' = (pK_1' + pK_2')/2$$

while for the type RNH₂(COOH)₂ the relation is

$$pM' = (pK_2' + pK_3')/2$$

For all of the amino acids considered by Cohn the pH of maximal charge lies between 5 and 7 (except for histidine, where it is 3.9). Cohn hints that this fact may have some physiological significance.

The constants of several peptides have been determined by Simms (22) and Greenstein (23) as a step towards the interpretation of the titration curves of proteins. Some of their values are given in Table II. It is evident that the pK' values of the peptides are closer together than those of the corresponding simple amino acids. This means, in terms of the Zwitterion theory, that the introduction of the peptide linkage has weakened the ionization of both acidic and basic groups. This effect is rather strikingly shown in Figs. 2 and 3, in which the solid circles represent the ionization of the dipeptides of histidine and of aspartic acid (23). For each peptide the whole course of the ionization takes place within a narrower zone of pH values than is the case with the component amino acids. Because of this compression of the range of pK values, only one of the four segments of each peptide curve is separated from the others, which have become fused into a single smooth curve covering the region in which three types of dissociation are effective. The absence of a break at the isoelectric points, pH 7.3 and 3.0, respectively, indicates that the isoelectric point of each of these peptides is sharply defined.

The influence of chemical structure on dissociation constants has attracted many workers (29, 30, 31). The simple relation proposed by MacInnes (31) has been found to apply to ionization constants of acids having an amino group in the α -, β -, γ -, δ - or ϵ -position (32, 33). This relation may be written

$$pK = pK_{\infty} + \frac{S}{d}$$

Here pK_{∞} and S are empirical constants and d is a measure of the distance between the amino and carboxyl groups, being 1 for the α -position, 2 for the β -position, etc. Recently Greenstein (34) has found that a modified relation holds even better. He replaces MacInnes' d by l^2 , but his l is an actual distance calculated from x-ray data. Other work in this field is discussed by Cohn (9).

- (10) Applications of Dissociation Data. Analytical methods for the determination of amino acids in solution include acidimetric titrations with indicators in the presence of formaldehyde, alcohol or acetone. These methods, which may be explained by changes in pK values produced by different solvents, are discussed in Chapter IV. Foster and Schmidt (35) made use of dissociation data in the separation of amino acids from protein hydrolyzates by electrical transport through membranes in a three-compartment cell. By the proper adjustment of pH they were able to separate the dibasic and diacidic amino acids from the others. It is expected that eventually the dissociation data for amino acids and peptides will make possible the interpretation of protein titration curves, but this application is still incomplete.
- (11) Evidence for the Zwitterion Theory. Since the publication of Bjerrum's paper in 1923 (7), it has been generally agreed that the natural amino acids, in the isoelectric condition, exist largely as amphions and not as undissociated molecules. One type of evidence which favors this view is the finding of Harris (28) that formaldehyde influences the titration curves of amino acids with alkali but not those with acid. Since a similar influence was observed with ammonia, but not with acetic acid, it was concluded that the alkali removed hydrogen ion from the amino and not from the carboxyl group, in agreement with the Zwitterion theory.

Another argument involves a comparison of the values of the apparent heat of dissociation (36, 37), which can be obtained from the variation of pK values with temperature. Reduced to its simplest terms, the argument is that temperature has relatively little influence on the acid and basic dissociation constants of non-amphoteric weak electrolytes, while it has a marked influence on the ionization of water. It is found (25) that pK_1 for a simple amino acid such as alanine is decreased only by 0.03 between 20° and 45°, while pK_2 is decreased by 0.63. The value of pK_w decreases by 0.78 over the same interval. On the amphion theory pK_1 refers to the carboxyl group, and its value changes little with temperature. On the same theory pK_B , which is $pK_w - pK_2$,

refers to the basic ionization of the amino group, and in this case it is decreased by 0.15 for a temperature increase of 25°. On the classical theory the acid constant of the carboxyl group would be pK_2 , which changes by 0.63, and the basic constant for the amino group would be $pK_w - pK_1$, which changes by 0.75. If it is admitted that the constants of the acid and basic groups in an amino acid should change less with temperature than does pK_w , then the amphion theory must be adopted.

A quantitative comparison of the influence of temperature on the ionization of weak electrolytes may also be made in the following way. Harned and Embree (38) have found that the ionization constants of many such substances vary with temperature according to the general equation,

$$pK - pK_m = f(t - t_m)$$

in which K_m is the maximum value of K which it has at some temperature t_m and f is a general function. For all of the substances which they considered, this function was found to be, as a good approximation,

$$pK - pK_m = 5 \times 10^{-5} (t - t_m)^2$$

This equation applied equally well when t_m and K_m had been determined experimentally and when they were outside of the conditions under which the experiments were carried out. If this equation is generally valid, the variation in pK with temperature must be the same for all substances if the comparison is made at corresponding temperatures, equidistant from t_m for each substance, for

$$\frac{d(pK)}{dt} = 10^{-4} (t - t_m)$$

This means that differences in the ionization of different types of electrolytes will appear, not in d(pK)/dt nor in the heat of ionization, but in the values of t_m .

The values of t_m for acetic acid and its homologues are in the vicinity of 20° to 25°, while t_m for ammonium hydroxide, treated as a base, is at about 40° (4). Water, however, is calculated to have its maximum ionization at about 360°. (Noyes observed it at 275°, but the experiments had to be done under high pressure.)

If the recent accurate data for alanine (25) are treated in this way, the equation fits the Zwitterion constants K_A and K_B , giving t_m as 43° and 89°, respectively. If the same data are translated into

classical constants, by using the proper value of K_w for each temperature, the equation fits equally well, but t_m is calculated to be 329° for k_b and 286° for k_a . Similar results may be obtained with the constants for glycine (26). Evidently these figures are best interpreted by assuming that the classical constants include the ionization of water, and that the true acidic and basic constants for these amino acids are the *Zwitterion* K_A and K_B of Bjerrum (7).

Other types of evidence for the Zwitterion theory are discussed in Chapters XVI and XVII.

2. AMPHOTERIC PROPERTIES OF PROTEINS

Direct evidence that proteins can combine with hydrogen or hydroxyl ions was obtained in 1898 by Bugarszky and Liebermann, who made use of the hydrogen electrode in protein solutions containing acid or base. Soon after Hardy (11) had stated his conception of the isoelectric point, he ascribed amphoteric properties to the colloidal particles of serum globulin, which he called pseudoions (39). The idea that the electrical charge of protein particles was due to ionization of the protein ampholyte was expressed by Jacques Loeb in 1904. While some colloid chemists (40, 41) still maintain that protein particles acquire charges by adsorbing hydrogen or hydroxyl or other ions, most workers in this field accept the idea that proteins are true amphoteric electrolytes, in agreement with Osborne (42), Sörensen (43), Robertson (44), Pauli (45), Loeb (46), Henderson (47), and Cohn (9).

(1) Isoelectric Points of Proteins. Whatever theory may be adopted, the fact remains that each protein has a definite isoelectric point which is often more sharply defined than that of a simple amino acid. Michaelis (68, 62, 50) used the method of electrical migration to determine the isoelectric points of a number of proteins, and he showed that a slightly soluble protein is least soluble at its isoelectric point. Pauli (45) found that minimum viscosity and minimum ionization were associated, and Sörensen (43) recognized that the apparent osmotic pressure of a protein solution containing electrolytes was least at the isoelectric point. Loeb (46) showed that the occurrence, at the isoelectric point, of minima in osmotic pressure, membrane potential difference, swelling of gelatin particles, and viscosity of gelatin solutions could be explained on the basis of Donnan's theory of membrane equilibrium. As a result of these findings, the isoelectric points of some proteins have

been determined by the location of minima in some of their properties, and not directly by the method of electrophoresis.

In Table III are given values for the isoelectric points, in pH units, of some of the better characterized proteins. Most of the values included in the table were obtained by one of the direct

TABLE III

Isoelectric Points of Proteins

Protein	Source	Method	pI'	Ref.	
Recrystallized proteins:					
Edestin	Seed, hemp	F., C., S. ¹	5.5-6.0	(50, 51, 52)	
Egg albumin	Eggs, hen	I.I.P., C.	4.84-4.90	(13, 53)	
Hemocyanin	Blood, snail	M.B.	5.05	(54)	
Hemoglobin, reduced	Blood, horse	M., I.I.P.	6.79-6.83	(55, 56)	
Hemoglobin, oxy-	Blood, horse	I.I.P.	6.7	(56)	
Insulin	Pancreas, beef	C.	5.30-5.35	(57)	
Lactoglobulin (frac-					
tion of "lactalbumin")	Milk, cow	s.	4.5-5.5	(58)	
Pepsin	Gastric mucosa,				
그리 구작을 받았으니 하니	swine	S., I.I.P., C.	2.75-3.0	(59)	
Serum albumin	Blood, horse	M.B.	4.88	(54)	
Trypsin	Pancreas, beef	C., F.	5.0-8.0	(60)	
Urease	Seed, jack bean	S.	5.0-5.1	(61)	
Amorphous proteins:					
Bence-Jones protein	Urine, human				
	(diseased)	M.B.	5.20	(54)	
Casein	Milk, cow	M., F.	4.6	(62)	
Gelatin	Skin, calf				
	(limed)	C., I.I.P.	4.80-4.85	(63)	
Gliadin	Seed, wheat				
	(flour)	S., I.I.P.	6.5	(64)	
Myogen	Muscle, rabbit	M.	6.2 - 6.4	(65)	
Myosin	Muscle, rabbit				
	and cow	I.I.P.	6.2-6.6	(66)	
Protamins	Sperm, fishes	I.I.P.	12.0-12.42	(67)	
Serum globulin	Blood, horse	M., F.	5.4-5.5	(68)	
Silk fibroin	Silk	M., S., V.	2.0-2.4	(69)	

¹ Abbreviations:

C. = Cataphoresis (micro)

F. = Flocculation maximum

I.I.P. = Isoionic point

M. = Migration (macro, qualitative tests)

M.B. = Moving boundary (photographic record)

S. = Solubility minimum

V. = Viscosity minimum

² The limits 12.0–12.4 include the values for protamins from the sperm of nine different kinds of fishes. Three other varieties yielded the values 9.7, 10.0 and 11.7.

electrical methods. "Migration" refers to Michaelis's method of electrophoresis in a U-tube with three compartments, the presence or absence of migration being determined by qualitative tests for protein in the end compartments. "Cataphoresis" refers to the motion of suspended particles in an electric field in a small cell observed under a microscope; often the particles were of some inert material, such as nitrocellulose or quartz, which adsorbed the protein. "Moving boundary" refers to the elaborate method of Svedberg and Tiselius, who obtained photographic records of the boundaries of the protein solution at different times during the passage of the current. "Isoionic point" refers to the pH of a solution in which hydrogen and hydroxyl ions are equally combined with, or dissociated from, the protein; the isoionic and isoelectric points are identical if the protein reacts with no other ions (13).

Other tables of isoelectric points have been given by Lloyd (48), Pauli and Valkó (45), and Thomas (49), who has made a very extensive compilation.

The figures in Table III show that most proteins are isoelectric at acidities somewhat greater than those of neutral solutions or many physiological fluids. A conspicuous exception is the enzyme pepsin, which is isoelectric in more acid solutions, near the region of its greatest catalytic activity. In the case of trypsin, too, there seems to be some correspondence between the isoelectric zone and the region of optimum activity, in agreement with a theory of Michaelis (12). The fact that protamins are isoelectric in strongly alkaline solutions has been of use in clinical work; at the pH of blood a protamin is positively charged while insulin is negatively charged, and the two substances unite to form a slightly soluble complex which exerts its physiological activity in the human body for a longer time than does insulin alone (70).

(2) Dissociation Curves of Proteins. By the use of the hydrogen electrode in protein solutions containing varied amounts of acid or alkali, it is possible to obtain electrometric titration curves, just as in the case of the amino acids. By subtracting the concentration of free hydrogen or hydroxyl ions from the total concentration of the strong acid or base used, one obtains a value for the amount of hydrogen or hydroxyl ion bound by the protein. The latter is equivalent to hydrogen ion removed from the protein. It is necessary to make an assumption in order to obtain ion concentrations from pH values; the assumption which has yielded the most reasonable results is that the individual ionic activity coefficient of H⁺

or OH- is not influenced by the presence of the protein, being the same as in an equally concentrated solution of the strong acid or base without protein.

The amount of a strong acid or base which is combined with a protein in aqueous solution may be calculated quite simply from measurements of the electromotive force of a concentration cell with two hydrogen electrodes (44, 9). For the titration with acid the cell is

The total molality, A, of the hydrochloric acid is the same in both of the solutions. The electromotive force is given by the Nernst formula,

$$E = k \log \frac{a_{\rm H}^{+*}}{a_{\rm H}^{+}}$$

on the assumption that the salt bridge eliminates liquid junction potentials. Here k is 2.3026 RT/F, and the asterisk refers to the protein-free acid solution. On the assumption that $\gamma_{\rm H}^+$ has the same value in both of the solutions, the activity ratio may be replaced by a ratio of molal concentrations, $[{\rm H}^+]^*/[{\rm H}^+]$. Since the acid is believed to be completely ionized, $[{\rm H}^+]^*$ is identical with A. If there are g grams of protein per kilogram of water, and if 1 gram of protein binds x equivalents of hydrogen ion, $[{\rm H}^+]$ may be replaced by A-gx; that is,

$$E = k \log \frac{A}{A - gx}$$

For the titration with alkali the cell is

H₂, NaOH (B), KCl (satd.), protein + NaOH (B), H₂ (+) Its electromotive force is given by the equation,

$$E = k \log \frac{a_{\mathrm{H}^+}}{a_{\mathrm{H}^+}^*}$$

if it is assumed that liquid junction potentials are cancelled. For dilute solutions the activities of water in the two solutions may be taken as identical, and the ion product law, equation (1), makes it possible to replace the ratio of hydrogen ion activities by the inverse ratio of hydroxyl ion activities, $a_{\rm OH}^{-*}/a_{\rm OH}^{-}$. If $\gamma_{\rm OH}^{-}$ has the same value for both of the solutions, the ratio becomes one of molal concentrations, $[{\rm OH}^{-}]^*/[{\rm OH}^{-}]$. Since sodium hydroxide is

a strong electrolyte, $[OH-]^*$ may be replaced by B, the total molality of the base. If 1 gram of protein in the alkaline solution loses z equivalents of hydrogen ion to form water (or binds z

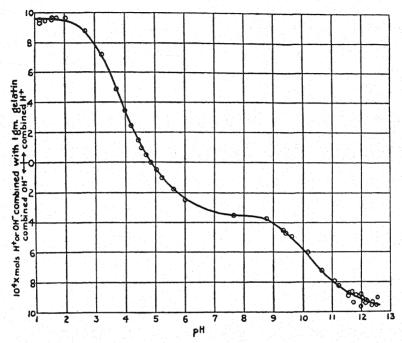


Fig. 6. Dissociation curve of a standard gelatin preparation in hydrochloric acid or sodium hydroxide solution, as obtained from pH measurements at 30°. The curve was drawn to fit the experimental points.

(Hitchcock, D. I., J. Gen. Physiol., 15, 125 (1931).)

equivalents of hydroxyl ion), the free hydroxyl ion in the protein solution is B-gz. The equation therefore becomes

$$E = k \log \frac{B}{B - gz}$$

It is thus possible to calculate the values of x and z, the numbers of equivalents of hydrogen ion bound or given off by one gram of protein, without using numerical values of $\gamma_{\rm H}^+$, $\gamma_{\rm OH}^-$, or K_w .

It has been customary to plot the results of such experiments against pH. If pH values are required, they may be obtained by measuring the potential between each half cell and a standard half cell containing a hydrogen electrode in a solution of known pH, or against a standard calomel half cell. If values of γ_H^+ , γ_{OH}^- , and

 K_w are to be used, they should be obtained in this way. Attempts to use values obtained in other ways have sometimes led to conflicting estimates of the combining capacities of proteins.

A combination curve which was obtained in this way is reproduced in Fig. 6 (71). The protein used was a gelatin preparation, made according to certain standard specifications. Fig. 6 is typical of the results obtained with other soluble proteins such as egg albumin (9, 72) and serum albumin (9). No break is evident at the isoelectric point; this is in agreement with the fact that such proteins have sharply defined isoelectric points. A protein insoluble at its isoelectric point, such as casein or edestin, combines with very little acid or base over a rather broad range of pH values; such combination curves have a very small slope in the isoelectric region (9). Several proteins which are isoelectric at about pH 5 show some flattening of the curve near pH 8. Such a flattening seems to mean that one set of ionizable groups becomes completely neutralized, or has given off all of its available hydrogen ions, in this region.

A comparison of Fig. 6 with the dissociation curves of amino acids and peptides (Figs. 1, 2, and 3) shows a marked resemblance between the curve for the protein and that for the quadrivalent peptide, aspartyl-aspartic acid. Such a resemblance was predicted by Henderson (47) on theoretical grounds before the ionization of multivalent peptides had been experimentally investigated. The principal difference is that this peptide has its isoelectric point at a low pH value, since it has three carboxyl groups and only one amino group. A protein molecule certainly has many more than four ionizable groups, the number being of the order of 30 to 60 for egg albumin (13, 72). The dissociation curves of proteins, however, are like that in Fig. 6 in having very few sections which are noticeably separated by points of inflection. Cohn (9) found it possible to reproduce the hydrogen ion dissociation curves for gelatin, casein, and egg albumin, within the experimental error of the data available in 1925, by theoretical curves calculated, in each case, on the basis of only three, four, or five pK values. Cohn (73) has recently pointed out that the values of these constants are remarkably close to those which were later found by Greenstein (23) to be characteristic of trivalent and quadrivalent peptides. This resemblance seems to be a result of the same compression of pK values which has been noted in comparing the values for the amino acids and peptides.

In the dissociation curve of an amino acid or a peptide, each section may be referred to a single group capable of dissociating or adding hydrogen ion. The maximum gain or loss of hydrogen ions in each section of the curve is therefore one equivalent per mole. In the case of a protein such a simple relation is not obvious because the constants lie so close together that each section covers the dissociation of considerably more than one group. Thus in Fig. 6 the maximal amount of acid bound by the protein is 0.96 milliequivalents per gram, while on the alkaline side hydroxyl ion is bound, or further amounts of hydrogen ions are dissociated, in steps, corresponding to 0.36 milliequivalents at about pH 8, and 0.54 milliequivalents more at pH 12. The fact that these numbers do not appear to be in the ratio of small integers may be explained by the large numbers of groups which are involved in each step in the titration, and it is not at all inconsistent with a simple chemical explanation of these hydrogen ion dissociation curves of proteins.

It is generally agreed that the central portions of the combination curves of proteins represent a truly reversible dissociation or addition of hydrogen ions. Sörensen, Linderström-Lang and Lund (13) were doubtful whether egg albumin retained its genuine qualities at acidities greater than pH 4, but Kekwick and Cannan (72) concluded that the titration of this protein was reversible between pH 2.5 and 11.0. There seems to be no doubt of the chemical stability of proteins, and of the reversibility of their dissociation, in the region of physiological acidities. The hydrogen ion dissociation curves of oxyhemoglobin and reduced hemoglobin in this range have been carefully studied by Van Slyke and his co-workers (56), and the results have been used to explain the distribution of gases and ions in arterial and venous blood.

(3) Determination of Acid and Base Combining Capacities. (See also Chapter XIII.) It may be seen that those points in Fig. 6 which lie between pH 1 and 2 are fairly well represented by a horizontal line, and that the points near pH 12 lie on a curve which seems to be approaching a horizontal asymptote as the alkalinity is increased. If these portions of the curves were definitely horizontal, it would indicate that the protein had attained a maximal capacity for combining with acid or base; that is, for adding hydrogen ion or losing it by dissociation. Electrometric studies of solutions of proteins in hydrochloric acid have led the writer (51, 71) to believe in the reality of a maximal capacity for binding hydrogen ions under these conditions. Cohn (9) in 1925 recalculated all of the

available data on the combination of proteins with acid and with base, and concluded that proteins in general showed such a maximal combining or dissociating capacity, both in acid and in alkaline solutions. Some workers (41) are not yet convinced that this point has been definitely established.

The experimental solution of this problem is rather difficult. Fig. 1 shows that glycine would not attain its maximal combination with hydrogen ions unless the pH value of the solution were below zero. Even in the titration of the multivalent ampholytes. Figs. 2 and 3 do not show a flattening of the curves above pH 1.0. and the experimental data did not extend so far into the region of high acidity. On the alkaline side the points in Figs. 2 and 3 lie quite definitely on the theoretically horizontal parts of the curves: the attainment of these maxima was possible because of the shorter range of pK values characteristic of multivalent ampholytes. Unless rather high concentrations of the ampholyte are used, the scattering of the points for regions of high acidity and alkalinity is worse than that shown in Fig. 6, because the amount of bound acid or base may be a small difference between two larger numbers. the amounts of the total and free acid or base. If the values adopted for $\gamma_{\rm H}^+$, $\gamma_{\rm OH}^-$, and K_w are not consistent with the method used, the results at extreme pH values may be markedly in error. If the experiments and calculations are made with due regard for these difficulties, it is possible to show that proteins do exhibit maximal combining capacities both in acid and in alkaline solutions. A good example of this is the curve for carboxyhemoglobin obtained by Cohn and his coworkers (91).

The combination of proteins with hydrochloric acid has been extensively studied by the writer, not only by the usual method of using the hydrogen electrode with a potassium chloride junction, but also by the use of a cell without liquid junction (74) of the type:

This double cell is a concentration cell without transport, and its electromotive force is given by the exact thermodynamic equation,

$$E = k \log \frac{a_{\text{H}}^{+*} a_{\text{Cl}}^{-*}}{a_{\text{H}}^{+} a_{\text{Cl}}^{-*}} = k \log \frac{A^{2} \gamma^{*2}}{[\text{H}^{+}][\text{Cl}^{-}] \gamma^{2}}$$

Here k is 2.3026 RT/F, A is the total molality of the acid, which is the same in both halves of the cell, γ is the mean activity co-

efficient of the ions of hydrochloric acid, and the asterisk refers to the protein-free acid solution. In order to interpret the results in terms of combining capacities, it is necessary to go beyond thermodynamics and make an assumption about the activity coefficients. Several alternative sets of assumptions are possible.

(I) The simplest assumption is that the value of γ is not changed by the protein and that hydrogen ion but not chloride ion is bound by the protein. The equation then becomes

$$E = k \log \frac{A}{A - qx}$$

where g is the protein concentration in grams per kilogram of water and x is the number of equivalents of hydrogen ion bound by one gram of protein.

(II) It might be assumed that the values of γ and γ^* were still identical, but that the protein combined not only with hydrogen ion but also with chloride ion. These assumptions lead to the equation,

$$E = k \log \frac{A^2}{(A - gx)(A - gy)}$$

if y is the number of equivalents of chloride ion bound by one gram of protein. This equation may be solved for x and y if two or more measurements are made with such solutions that E and g (or A) are different but x and y are constant.

(III) Another possibility is that a protein in acid solution combines with hydrogen ion but not with chloride ion, and that the value of γ is less than that of γ^* , owing to the partial replacement of the hydrochloric acid by an ionized protein hydrochloride. Such a decrease in γ is indicated by the linear law of Güntelberg (75) and of Harned (76), who measured the values of γ for hydrochloric acid in the presence of other univalent chlorides at a constant total molality of chloride. This law may be expressed by the equation,

$$-\log \gamma = -\log \gamma^* + \alpha m$$

in which m is the molality of the salt and α is a constant. A similar relation was found by Failey (77) to hold for the effect of the protein edestin, in solution in dilute nitric acid, on the activity coefficient of thallous chloride. If the protein hydrochloride has an effect similar to that of an added univalent chloride, the linear

law may be written

$$-\log \gamma = -\log \gamma^* + bg$$

in which b is a constant, since the equivalent concentration of the protein salt will be proportional to the weight concentration of the protein if a maximum combining capacity for hydrogen ion has been attained. On the basis of these assumptions the electromotive force of the cells is given by the relation

$$E = k \log \frac{A}{A - gx} + 2 kbg$$

This equation may be solved for x and b, if these quantities are constant, by the use of two or more measurements with varied values of g (or A) and E.

Table IV shows some of the results of these measurements for three proteins, calculated by the three sets of assumptions. In each case Assumption I gives values of 10^3x which decrease as the concentration of protein is increased, and the values are all higher than those which had been obtained previously by the use of cells with a salt bridge, which are about 0.6 to 0.9 for casein, 1.25 to 1.45 for edestin, and 0.96 for this gelatin preparation (78, 9, 71). Assumptions II and III, on the other hand, yield remarkably constant combining capacities with varying protein concentrations. The values of x given by these two assumptions are in excellent agreement with each other, and in fair agreement with the older values just given. Assumption III seems at present most likely to be correct, as it is in accord with the results which have been obtained with solutions containing both acid and salt.

This method has not yet been applied to the study of the combination of proteins with alkali. The usual pH method has in most cases yielded points which are more scattered, in the region of high alkalinity, than are those in Fig. 6. It seems possible that this uncertainty may be connected with the formation of carbamino protein compounds, due to the reaction of carbon dioxide with protein anions. The existence of such compounds in alkaline solutions of hemoglobin or serum proteins has recently been reported (79).

The significance of maximum combining capacities obtained from the ordinates at the ends of curves such as that of Fig. 6 may be questioned because of possible changes in the protein itself in strongly acid or alkaline solutions. Svedberg (80) has found, by measurements with the ultracentrifuge, that the stability of proteins, with respect to molecular weight or particle size, is limited to an intermediate range of pH values which does not include the extremes which are necessary to produce maximum combination

Table IV

Acid Combining Capacities of Proteins from the Electromotive Force of Cells without Liquid Junction at 30°

Protein	g = protein per kg. H ₂ O	$A = HCl$ per kg. H_2O	E = E.M.F of double cell	$10^3 x = H^+$	bound per g	m. protein
	gm.	moles	millivolts	1	milliequivaler	$_{ m nts}$
				1	II	III
Casein	20.0	0.1000	6.6	1.116	0.814	0.800
	30.0	0.0999	10.2	1.076	0.801	0.796
	40.0	0.0999	14.1	1.042	0.796	0.795
	50.0	0.1000	18.5	1.015	0.799	0.802
Edestin	18.55	0.1020	9.0	1.602	1.336	1.326
	42.35	0.1050	24.4	1.505	1.345	1.340
	62.7	0.1055	47.6	1.411	1.339	1.340
Gelatin	50.3	0.1000	19.5	1.046	0.957	0.958
	70.4	0.1000	32.8	1.016	0.961	0.961
	90.5	0.1000	57.1	0.981	0.958	0.959

Assumptions used in calculating x:

(I)
$$E = 60.15 \log \frac{A}{A - gx}$$
.

(II)
$$E = 60.15 \log \frac{A^2}{(A - gx)(A - gy)}$$
, where $10^3y = 0.36$ for casein, 0.35 for edestin, and 0.17 for gelatin.

(III)
$$E = 60.15 \left(\log \frac{A}{A - gx} + 2bg \right)$$
, where $10^4b = 8.5$ for casein, 8.0 for edestin, and 3.85 for gelatin. (Hitchcock, D. I., *J. Gen. Physiol.*, 16, 357 (1932).)

with acid or alkali. Ettisch and Schulz (81), in titrating serum proteins with alkali, found a slow change in pH for 24 hours or more; this they interpreted as due to hydrolysis of the protein. Similar drifts were noticed by Kekwick and Cannan (72) in titrating egg albumin below pH 2.0 and above pH 11.5. Yet the writer (74) found that cells without liquid junction, containing protein solutions of pH 1 to 2, gave electromotive force values which were

constant for many hours. This constancy seems to mean that, although the protein may be altered, it forms products which reach a steady state of ionization within a short time.

Another method of estimating combining capacities of proteins was introduced by Bancroft (82), who had long held the view that the combination of proteins with acid or base was not a stoichiometric chemical reaction but colloidal adsorption. He made phase rule studies of the combination of dry proteins with ammonia and hydrogen chloride gases. The method was tested with various crystalloidal organic substances, and the results with proteins led to the conclusion that certain proteins did form chemical compounds with hydrogen chloride but only adsorption complexes with ammonia. The amounts of hydrogen chloride combined were considerably greater than the amounts of hydrogen ion bound by the same proteins in solution, as given by other observers who had used the electrometric method. Similar experiments, with very carefully purified and dried amino acids and proteins, were carried out by Czarnetzky and Schmidt (83), who concluded that the proteins studied by them formed chemical compounds with ammonia as well as with hydrogen chloride (see Chapter XIII).

(4) Correlation of Combining Capacities with the Amino Acid Content of Proteins. Various workers, including Goto (84), Kossel and Cameron (85), Bracewell (86), and Greenberg and Schmidt (87), noticed that the maximum combining capacities of proteins for acids and bases were related to the amounts of the diamino and dicarboxylic acids contained in the proteins. Cohn (9) compiled a table showing a fair degree of parallelism between the acidbinding capacities of eight proteins and the total basic amino acids (histidine, arginine, and lysine) contained in each. A similar correlation was observed between the base-binding capacities of three proteins and the sum of the dicarboxylic acids (glutamic acid. aspartic acid, and β -hydroxyglutamic acid), less the amide nitrogen, contained in each. For casein the correlation could be improved by including tyrosine as a dibasic acid. A more extensive comparison was made by Czarnetzky and Schmidt (83), including the results of their study of the gas-binding capacities of dry proteins, as well as some earlier work of Schmidt and collaborators (88) on the combination of proteins with acid and basic dyes (see Chapter XIII).

It is not always possible to decide whether a certain group in a protein is ionized in solution on the acid or on the alkaline side of the isoelectric point. This is especially true for a protein whose dissociation curve shows no break at the isoelectric point. Cohn (9) has tabulated the total number of free ionizable groups of certain proteins. Cohn, Edsall and Blanchard (89) have indicated that the dissociation of zein may be explained by assuming that the free carboxvl groups in the protein are responsible for the part of the curve between the breaks at pH 3 and 8, although hydrochloric acid was bound at pH values below about 6.3 and sodium hydroxide was bound at higher values. It might therefore be reasonable to compare the sum of the acid and base binding capacities of any protein with the sum of the free ionizable groups, both acidic and basic, as calculated from the analytical data.

(5) Application of the Zwitterion Theory to Proteins. Isoelectric proteins are believed to exist largely in the form of Zwitterionen, for the same reasons advanced in the case of amino acids. Their behavior is similar in the formol titration. The changes in heat and in volume accompanying the reactions of a protein with a strong acid or base are more readily explained on the assumption that the addition of acid results in the formation of unionized carboxvl groups while the addition of alkali results in the removal of hydrogen ion from positively charged amino groups (36, 37). There is evidence that protein solutions have higher dielectric constants than water (73). Since the numbers of acidic and basic groups in a single protein are not, in general, exactly equal, an isoelectric protein will not have all of its groups ionized in the Zwitterion form. There will be some unionized carboxyl or amino groups, just as in isoelectric aspartic acid or lysine. If the isoelectric point is sharply defined there will also be a definite fraction of the protein ionized as cations and anions, even at the isoelectric point, just as in the case of a simple ampholyte with pK values close together. Such ionization would explain the finding that gelatin combined with certain acid dyes at pH values somewhat on the alkaline side of its isoelectric point (88).

3. LITERATURE

Early work in this field is covered by the monograph of Robertson (44), who was among the first to make extensive applications of physical chemistry to protein systems. Readers of his book should be warned, however, that his theory of the ionization of protein salts is no longer tenable. The book of Loeb (46) is an eloquent argument, based on his own experiments, in favor of a simple physico-chemical explanation of many properties of proteins; practically all of his conclusions are still valid. More useful as a reference book is that of Pauli and Valkó (45), who have covered the whole field of the colloid chemistry and physical chemistry of proteins, with numerous references to the literature. The papers of Sörensen (18, 16, 43, 13) are those of a master of this subject, but a beginner may find them difficult to read because of their wealth of detail. The valuable review articles of Cohn (9, 73) are indispensable to workers in protein chemistry.

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CHAPTER XII

ELECTROCHEMISTRY OF AMINO ACIDS AND PROTEINS

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1. INTRODUCTION

Experimental work which has been carried out over a period of several decades has indicated that aqueous solutions (and in some instances non-aqueous solutions as well) of the soluble salts of amino acids and proteins are fairly good conductors of the electrical current and hence are appreciably ionized. The significant point which has been gained from these studies is that the amino acids and proteins belong to the general category of electrolytes, and therefore obey the same chemical laws which have been found to apply to the group as a whole.

It is the aim of the present chapter to survey the more important electrochemical methods which have been employed in the study of solutions of amino acids and proteins, to present the results which have been obtained, and to interpret and evaluate the data.

2. ELECTRICAL TRANSPORT OF PROTEINS

It was first pointed out by Hardy (1) in 1899 that when a direct electrical current is passed through a slightly acid solution of denatured egg albumin, the protein is deposited on the cathode, indicating that the protein ions carry a positive charge; whereas, in the case of a slightly alkaline solution of egg albumin, the protein is deposited on the anode, showing that the protein ions are negatively charged. Serum globulin was found to behave similarly (2). It is very probable that all proteins which are insoluble at their isoelectric points will be deposited likewise. On the other hand, such proteins as gelatin and undenatured egg albumin, which are not insoluble in water at their isoelectric points, will not be deposited on a metallic electrode even though they may migrate under the influence of a direct current.

These experiments are of a purely qualitative nature. They indicate that the protein salt is ionized, that the ions migrate when a direct current is passed through the solution, and that the

direction of migration depends on the reaction of the solution with reference to the isoelectric point of the protein, i.e., whether the protein is playing the part of an acid or a base.

The proportion of the current which each of the ion species will carry depends on the nature of the ions which are present in the solution and their relative velocity. Since all of the ions do not migrate through the solution with the same velocity, it is obvious, other things being equal, that the faster moving ions will carry a larger proportion of the current than those which move more slowly. The number of equivalents of cation which move toward the cathode plus the number of equivalents of anion which move toward the anode must be equal to the total number of equivalents of electricity which have passed through the solution, or,

$$N_c + N_a = N_e. (1)$$

The fractions of the current which each of the ions carries are termed the transport numbers of the respective ions. Expressed mathematically,

$$\frac{N_c}{N_c + N_a} = \frac{N_c}{N_e} = T_c \tag{2}$$

and

$$\frac{N_a}{N_c + N_a} = \frac{N_a}{N_c} = T_a \tag{3}$$

where T_c and T_a are the transport numbers of the ions indicated by the subscripts. It is obvious that, unless complex ions are present in the solution, as a result of which a part of the ion species migrates in a direction opposite to that of the remainder, the sum of the transport numbers of the cation and anion, respectively, will be equal to unity.

Several methods are available for the determination of transport numbers. The moving boundary method of Denison and Steele (3) depends on the fact that the ratio of the transport numbers of the two ions in a solution of an electrolyte is equal to the ratios of the velocities with which the two ion-constituents move through the solution, and therefore equal to the ratio of the distance traversed by the two ion-constituents in a given time during which the current flows. In practice, a solution of the salt CA, which is being studied, is placed between a solution of salt C'A and salt CA'. The boundary between solutions CA and CA' will be designated as

aa, and that between solutions CA and C'A as bb. For purposes of this discussion we may consider that the three solutions are contained in a vertical tube with an electrode at each end, and the direction of flow of the current is downward. On passing a direct current through these solutions, boundary aa moves upward to a'a'. This is the distance which is covered by the anion-constituent A during the passage of the current. Similarly, boundary bb has moved downward to b'b'. The distance is that which is covered by the cation-constituent. The distances are usually measured refractometrically. The ratio of the two distances is the ratio of the two transference numbers.

The method of Denison and Steele has been tried on protein solutions in this laboratory, but, due to the fact that a sharp boundary was not obtained, it was abandoned in favor of the well-known Hittorf method (4). This method is based on the fact that, since the different ion species in a solution move with different velocities, on passing a direct current through the solution, changes in the composition of the electrolyte will take place in the region of the two electrodes. The amount of these changes will depend on the quantity of the current which has passed through the solution and the transport numbers of the ions. The changes in the composition of the solution in the vicinities of the electrodes are determined by chemical analysis.

We may illustrate the Hittorf method for determining transport numbers by means of a simple system. If a direct current is passed through a solution of silver nitrate which is contained in a threecompartment cell, silver electrodes being employed, it will be found that the concentration of silver nitrate in the anode compartment has increased. In the cathode compartment there will be a decrease. On passing N_e equivalents of electricity through the solution, N_e equivalents of silver will dissolve from the anodic electrode and pass into the solution as silver ions. At the same time, N_c equivalents of silver ions will move out of and $N_e - N_c$ equivalents of nitrate ions will move into the anode compartment. Let the composition of the anode compartment be N₂ equivalents of AgNO₃ in mw grams of water, and the composition of the solution before passage of the current be N_1 equivalents in m_w grams of water. The increase $N_2 - N_1$ must be equal to $N_e - N_c$, that is, it must be equal to the silver which has come into the anode portion from the electrode, diminished by the silver which has gone out of the anode compartment due to the migration of silver ions toward the

cathode. Hence, from equation (2), the transport number of the silver ion is

$$T_{c} = \frac{N_{c}}{N_{e}} = \frac{N_{1} - N_{2} + N_{e}}{N_{e}} \tag{4}$$

and that of the nitrate ion is $T_a = 1 - T_c$. At the cathode the reverse of the above process takes place. From analysis of the solution

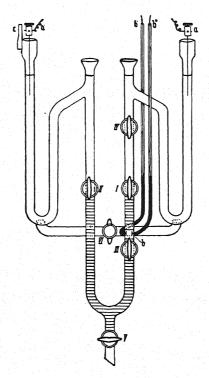


Fig. 1. Transport cell according to Engel and Pauli. (Pauli, W. and Valkó, E., Kolloidchemie der Eiweisskörper, Dresden and Leipzig, 1933, p. 151.)

contained in the cathode compartment a check on the two transport numbers can be made.

It is evident that the estimation of transport numbers of protein ions is not quite so simple as that which is indicated by the above illustration. This is due to the fact that it is not possible to employ reversible electrodes as it is in the case of silver nitrate solutions. The acidity of the solution must be kept within narrow limits to prevent resolution of the deposited protein. The special features

which are employed in estimating transport numbers of proteins are given in the succeeding paragraphs.

Various types of cells have been used in transport experiments. The apparatus which has been described by Engel and Pauli (5) is shown in Fig. 1. The electromobility of the protein ion is determined by a procedure which combines features common to both the moving boundary and the Hittorf method. The determination is carried out by filling the cell with the test solution to the level of stopcocks I and III. The rest of the cell above these stopcocks is

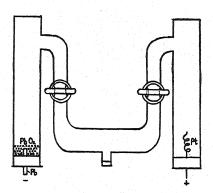


Fig. 2. Transport cell. (Greenberg, D. M., and Schmidt, C. L. A., J. Gen. Physiol., 7, 287 (1924).)

filled with a solution of an indifferent electrolyte, usually 0.1 N potassium chloride. A direct current is passed through the cell in the direction that will cause the ion under investigation to move towards stopcock IV. After a time stopcocks III and IV are closed and the rest of the apparatus is washed with distilled water. The solution remaining in the apparatus is analyzed. To determine that no change in composition of the test solution in the middle portion has occurred, the conductivity is determined at point b. For this purpose electrodes b'b' are sealed in. Knowing the initial and the final concentrations of the protein solution, the migration velocity of the protein ion is calculated with the aid of the equation,

$$U = \frac{X\left(\frac{a}{c} - V\right)}{Q} \tag{5}$$

where U = migration velocity of the protein ion in cm./sec./volt, X = the specific conductivity of the solution, a = the initial, and

TABLE I.

Calculation of Transference Experiments.

Sample A.

 $2.85~\rm per~cent~case in + 5.6~cc.~0.1~N~NaOH~per~gm.$ Temperature 30°. Time 2.5 hrs. Current 2.55 cc. 0.1 N Ag.

Anode Portion = 76 Gm.

Electrode deposit.

3.6240

3.1695

0.4545 gm.

Q (electrochemical equivalent) = 0.4545/0.255 = 1.78 gm. per millifaraday.

pH of solutions

Original =6.45

Middle = 6.50

Anode =6.50

Cathode = 6.45

Analysis (10 gm. Samples Used)

Anode	Anode	Middle	Original
13.2540	12.7550	15.0190	17.9913
13.0000	12.4998	14.7300	17.7035
0.2540	0.2552	0.2890	0.2878

Mean conc. = 2.546 per cent. Corrected for Na = 2.515 per cent.

Anode Gain of Casein.

Mean conc. = 2.884 per cent. Corrected for Na = 2.845 per cent.

(2.845-2.515) 0.76=0.250 gm.

0.4545 - 0.250 = 0.2045 gm. Transfer of casein per millifaraday.*

Anode Loss of Na (15.9-13.9) 0.76 = 1.52 cc. 0.1 N Na

0.2045/0.255 = 0.802 gm. $T_{\text{casein}}^- = 0.802/1.78 = 0.45$

 $T_{\rm case in}{}^- = 0.802/1.78 = 0.45 \qquad T_{\rm Na}{}^+ = 1.52/2.55 = 0.596$ Total.....(0.45+0.596) = 1.046

Alkali Analysis (Titration with Trichloroacetic Acid).

Amount	Sample	0.05 N acid 0	.1 N per 100 gm.
			sample
cc. 40	Middle	cc. 12.8	cc. 15.9
40	Anode	11.1	13.9

* Since each cc. of 0.1 N Ag liberated in the coulometer represents 0.1 millifaraday then 2.55 cc. is equivalent to 0.255 millifaraday.

(Greenberg, D. M., and Schmidt, C. L. A., J. Gen. Physiol., 7, 287 (1924).)

c = the final concentration of the protein ion, V = the volume of the space between stopcocks III and IV, and Q = the quantity of electricity in coulombs which has passed through the cell.

The cell which has been employed by Greenberg and Schmidt (6) is illustrated in Fig. 2. It has been extensively used in this laboratory for transport experiments (6). The cell consists of three

Table II

Transport Numbers (Average Values at 30°)

(a) Sodium Caseinate Solutions

Time	Casein	B aproxi- mately	pН	Q	K	Casein transferred per milli- faraday	$T_{ m casein}$	$T_{ m cation}$
hrs.	per cent	cc.						
2.5-3	1.75-3.0	5.6	6.6	1.73	9.68	0.784	0.453	0.561
2.5	2.5	7.0	7.0	1.34	9.40	0.610	0.455	0.540
2.5	1.7	8.25	7.6	1.04	8.60	0.450	0.430	
		(b) I	Potassiun	n Caseir	rate Sol	utions		
3	2-3	5	6.5	1.82	9.09	0.650	0.359	0.660
2.25 - 4.0	2.5-3	6.25	6.9	1.47	9.17	0.534	0.363	0.636
2-2.5	2-2.5	8.0	7.6	1.12	9.00	0.390	0.349	0.657
2.0	2.0	10.0	9.4	0.92	9.20	0.350	0.382	0.654
		(c)	Rubidiun	r Caseir	rate Sol	utions		
2.0	1.8	5.6	6.5	1.76	9.86	0.625	0.355	0.645
		(d)	Cesium	Caseino	ıte Solu	tions		
2-2.5	2.0	5.5	6.5	1.65	9.10	0.551	0.334	0.666
		(e) Sod	ium Rac	emic Ca	seinate	Solutions		
3-3.5	2.0	5.4	5.75	1.63	8.83	0.635	0.390	0.610
		(f) Pota	ssium Ra	cemic C	aseinat	e Solutions		
2.25-3.0		6.0	5.5	1.46	8.76	0.465	0.318	0.680
1.5 - 2.5	1.6	8.65	6.4	0.985	8.50	0.355	0.360	0.610

B = cc. 0.1 N alkali per gm. casein.

Q = electrochemical equivalent per millifaraday.

(Greenberg, D. M., and Schmidt, C. L. A., J. Gen. Physiol., 7, 287 (1924).)

compartments of approximately 60 cc. each, separated by stopcocks of large bore. Unless large bore stopcocks are employed, the electrical resistance of the protein solution is too high to permit passage of sufficient amount of current in order to bring the changes produced in the solution within the limits demanded for analytical measurement. In order that the current be carried only by the protein salt, any excess of acid or base beyond that which is required to hold the protein in solution should be avoided. A spiral of platinum wire or a strip of platinum gauze is used as anode. The cathode consists of Pb covered with PbO₂. This is only one of a number of possible non-polarizable electrodes. The use of a platinum anode, upon which the protein deposits, as a non-polarizable electrode is limited to proteins which are insoluble at their isoelectric points. The constancy of the pH of the solution is maintained by the two non-polarizable electrodes.

The amount of current which is passed through the solution may be determined by the use of the silver or iodine coulometer. The

Table III

Transport Data of Casein at Various Temperatures
(Average Values)

Protein	Dissolved in	Temperature	$T_{ m casein}$	T _{cation}	K
		degrees			
Casein	NaOH	7	0.365	0.61	9.9
		18	0.390	0.59	9.9
		25	0.425	0.62	9.55
		35	0.430	0.55	10.1
		40	0.440	0.55	10.1
		48	0.480	0.54	9.85
	LiOH	25	0.485	0.48	9.95
		30	0.495	0.495	10.2
		48	0.520	0.56	10.0
	KOH	48	0.385	0.57	9.8

(Greenberg, D. M., Univ. of Calif. Pub. Physiol., 7, 9 (1927).)

amount of protein which is deposited on the anode is determined by weighing. Analysis of the protein content of the solutions in the three compartments may conveniently be made by evaporating aliquot portions to dryness and subtracting the weight of the alkali metal in the solution. The latter, in the case of alkali caseinate solutions, may be determined by titrating with trichloroacetic acid to the isoelectric point of the protein. The calculations of a typical transport experiment are given in Table I.

The amount of protein which is deposited at the electrode is a measure of the current which has passed through the solution. The total gain in protein in the anode or cathode compartment, divided by the weight of the deposit, represents the fraction of the current which the protein carries when in solution, or, the transport number of the protein. The transport numbers of the inorganic ions are determined on the basis of direct analysis of the protein-

containing solution. If protein ions and inorganic ions are the sole carriers of the current, the sum of their respective transport numbers should be unity. The data which are given in Table II are, within the limits of the experimental error, in agreement with this statement.

The interesting fact which the data in Table II show is that the quantity of casein which was deposited on the platinum electrode is proportional to the current which has passed through the solution and, within limits, inversely proportional to the amount of alkali which is combined with the protein or to the charge which the protein carries. The facts can be expressed by a generalized form of Faraday's law¹ of electrodeposition (6, 7):

$$B \times Q = K$$
, (6)

where B = the number of cc. of 0.1 N alkali which is combined with each gram of protein, Q = the electrochemical equivalent per millifaraday, and K is a constant. Equation (6) likewise applies to proteins which are dissolved with the aid of acids (8).

The data which are given in Table II show that the value for Q is the same for solutions with equivalent amounts of alkali per gram of protein and is independent of the nature of the alkali hydroxide which is used to dissolve the protein. Within certain limits, the values for Q vary with the amount of alkali which is added to the protein. In regions of rather high alkalinity the value which is found for K may be somewhat less than when the solutions are near the neutral point. This is due to the experimental error which results from resolution of the protein deposit on the electrode by the alkali. The transport number of the protein varies with the mobility of the cation with which it is combined. When dissolved in a solution of lithium hydroxide, casein carries the larger fraction

$$F = \frac{Ne}{q}$$

where F=the Faraday constant, q=quantity of electricity which has passed through the solution, and Ne=equivalents of chemical change which is produced during the passage of q amounts of electricity. N is Avogadro's number and e is the charge carried by one electron. F=96500 (\pm 0.01 per cent) coulombs per equivalent.

¹ Faraday's law may be stated as follows: the amount of a chemical change or changes, expressed in chemical equivalents, which occurs when a direct electric current passes across a junction between a metallic and an electrolytic conductor, is proportional to the quantity of electricity which passes. This statement is expressed by the equation

of the current, since the lithium ion possesses a relatively small mobility. With ions of larger mobility the amount falls off in the order of sodium, potassium, rubidium, and cesium.

That the amount of protein which is deposited on the electrode is really a measure of the current which has passed through the solution is shown by the following. If all of the acid or base which is contained in the solution is combined with all of the protein,

TABLE IV

Transport Numbers of Some Protein Solutions at 25°
(Averaged Values)

Protein	Dissolved in	pH	K	T _{protein} -	$T_{ m cation}$
Casein	LiOH	6.5-7.2	9.95	0.485	0.48
Casein	NaOH	6.5-7.0	9.55	0.425	0.62
Casein	кон	6.5-9.4	9.10	0.360	0.65
Fibrin A	NaOH	9.5-10.5	5.95	0.49	0.47
Fibrin B	NaOH	9.5-10.0	9.2	0.48	0.52
Fibrin C	NaOH	9.5	7.45	0.46	0.50
Fibrin C	кон	10.0	8.0	0.37	0.58
Protein	Dissolved in	pН	K	$T_{ m protein}$ +	$T_{ m anion}$
Fibrin A	HCI	3.5	7.2	0.50	0.50
Fibrin C	HBr	3.5	4.95	0.48	0.63
Fibrin C	HNO ₃	3.5	6.1	0.55	0.36
Fibrin C	H ₈ PO ₄	3.3-2.85	4.35	0.89	0.23
Fibrin C	нсоон	3.5	4.8	1.28	0.34
Fibrin C	C ₃ H ₆ O ₃	3.5	6.3	1.29	0.28

(Greenberg, D. M., Trans. Amer. Electrochem. Soc., 54, 107, (1928); J. Biol. Chem., 78, 265 (1928).)

each unit of protein carrying its proportionate share of the charge, and, assuming a current efficiency of 100 per cent, the value of K in equation (6) should be 10 and cannot exceed this (7, 8). In the case of casein it has been found experimentally, at various temperatures, and using various members of the alkali hydroxides, that the value for K is very nearly 10 (see Table III). However, in the case of fibrin, it has been shown by Greenberg (8) that the value of K depends on the method which is employed in the preparation of this protein. Each fibrin preparation yielded a characteristic value for K and all values were less than 10 (see Table IV). The phenomenon is explained on the assumption that two or more

species of fibrin, which have different charges, are present in the solution. The molecules which carry the higher charge have a greater tendency to be deposited on passage of the current.

The transport numbers which have been obtained (9) in casein solutions as concentrated as 8 per cent and at an equivalent cation concentration up to 0.08 N are no different than in dilute casein solutions. The inference is that such protein solutions are completely ionized in the sense that this term is used in present day theories of strong electrolytes.

3. MOBILITIES OF PROTEINS FROM TRANSPORT EXPERIMENTS

The transport numbers of the two constituents of a binary electrolyte are related to each other according to the following equation:

$$\frac{T_{\text{(anion)}}}{T_{\text{(cation)}}} = \frac{\Lambda_{0\text{(anion)}}}{\Lambda_{0\text{(cation)}}} \tag{7}$$

where Λ_0 is the mobility or equivalent conductance at infinite dilution. For purposes of determining the value for $\Lambda_{0(anion)}$, the transport numbers of the anion and of the cation, respectively, are determined experimentally. Data for the mobility of the particular cation used may be obtained from International Critical Tables.

Table V

Mobility of Casein Ion at Different Temperatures

Cation used	Temperature degrees	$T_{ m casein}$	$\Lambda_{ m cation}$	$\Lambda_{ m casein}$
Sodium	7	0.365	31.8	18.2
Sodium	18	0.390	43.5	28.0
Sodium	25	0.425	51.1	37.9
Lithium	25	0.485	39.7	37.3
Sodium	30	0.455	56.2	46.2
Potassium	30	0.365	81.4	46.5
Cesium	30	0.335	85.3	43.0
Rubidium	30	0.355	84.5	45.5
Lithium	30	0.495	43.3	42.5
Sodium	35	0.430	61.5	47.0
Sodium	40	0.440	66.9	52.5
Sodium	48	0.480	75.4	69.5
Lithium	48	0.520	59.9	65.0
Potassium	48	0.385	106.6	66.5

(Greenberg, D. M., Univ. Calif. Pub. Physiol., 7, 9 (1927).)

The mobility of the protein ion can now be calculated by substituting the known values in equation (7). Values for the mobility of the casein ion at 30° are given in Table V. The data indicate that, within the limits of the experimental error, the mobility of the casein ion is independent of the cation with which it is combined. The comparatively high value for the mobility of the casein ion is

Table VI

Mobility of Fibrin in Alkali and Acid Solutions Calculated from Transference Data
Temperature 25°.

Cation used	$T_{ m fibrin}$	$T_{ m cation}$	$\Lambda_{0 { m cation}}$	Ao fibrin-
Sodium	0.470	0.50	51.1	45.3
Potassium	0.375	0.58	74.8	44.7
Lithium	0.515	0.44	39.7	42.3
Average				44.0
Anion used	$T_{ m fibrin}$ +	Tanion	$\Lambda_{0 ext{anion}}$	A o fibrin+
Chloride	0.51	0.50	75.8	78.7
Bromide	0.50	0.45	77.8	77.8
Nitrate	0.55	0.36	70.6	86.0*
Average				78.0

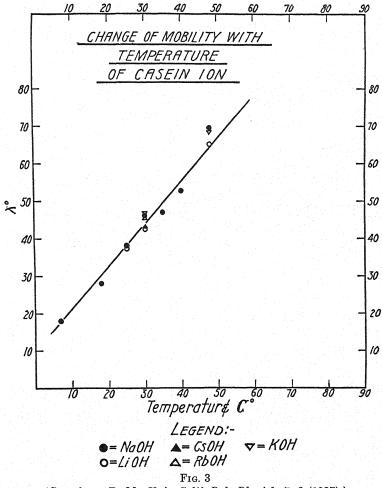
^{*} Value not used in obtaining average. (Greenberg, D. M., J. Biol. Chem., 78, 265 (1928).)

noteworthy. At 30° the value is about 45.3 mhos which is higher than that for the lithium ion (43.7 mhos). It is about the same as that of the acetate ion (45 mhos).

The mobility of the fibrin ion (see Table VI), as would be expected, is independent of the mode of preparation of this protein since the transport numbers of all preparations are essentially the same. The average value for the mobility of the fibrin anion, 44, does not differ materially from that of the casein ion. The mobility of the fibrin cation, 78, is of the same order as that of the potassium ion.

A discussion of other methods of determining mobility will be given later in this chapter.

Greenberg (9) has shown that, between 5° and 50°, the relation between the mobility of the casein ion and the temperature is a



(Greenberg, D. M., Univ. Calif. Pub. Physiol., 7, 9 (1927).)

linear one (see Fig. 3). The change of casein ion mobility with temperature is expressed by the empirical equation,

$$\Lambda_{0c} = 1.12 \ t + 9.7 \tag{8}$$

or 1.12 mhos per degree. If the temperature coefficient of the mobility at 18°, as defined by Kohlrausch, $\frac{1}{\Lambda_{0_{18}}} \cdot \frac{d\Lambda_{0}}{dt}$ is calculated for casein from equation (8), the value 0.0375 is obtained. This value is very much higher than the value 0.0265, the temperature coefficient of the lithium ion, which possesses the highest temperature coefficient of the inorganic ions. The significance of the high value for the casein ion is not clear.

4. TRANSPORT NUMBERS OF COMPLEX PROTEIN-CONTAINING IONS

Greenberg and Schmidt (10) have shown (see Table VII) that, when casein is dissolved in solutions of the alkali earth elements, the transport number of the casein ion is abnormally high; in some instances the value is greater than 1.0. The results indicate the presence of complex metallic-containing casein ions in these solutions. Some of the metallic element is carried in a direction which is opposite to its normal path. Hence unusual transport values are obtained. Complex ions are likewise formed (8) when fibrin is dissolved in solutions of formic, lactic, and phosphoric acid (see Table IV). The type of ionization which yields complex ions with, for example, the alkaline earth elements, is schematically expressed by the equation,

$$M_a \text{ protein} = xM^{++} + M_{(a-x)} \text{ protein}^{-2x},$$
 (9)

where M stands for the alkali earth element, a, the valency, and x, the amount of alkaline earth element dissociated. Equation (9) may be used to develop a formula for the transport number of the cation in terms of the fraction of the cation in the form of free ions and the mobilities of the ions in solution as follows:

$$T_{\text{cation}} = \frac{iu - (1 - i)v}{i(u + v)} \tag{10}$$

where i=fraction of ionized protein, u=mobility of the cation, and v=mobility of the complex ion. The value of u is obtained from International Critical Tables. No numerical data are available for the term, v. This value can be calculated with the aid of transport and conductivity data. Knowing the total conductivities of the alkaline earth protein solution, and the mobilities of the alkaline earth ion and of the protein ion, and the per cent ionization of the alkaline earth proteinate, the mobilities of the complex ion are calculated as the difference between the value of Λ and the sum of the mobility of the alkaline earth and the protein ion. The value for the mobility of the calcium-casein complex ion is 32.2, and that for the calcium-dephosphorized casein complex ion is 33.0.

From calculations which have been made with the aid of equation (10), it appears that, when 6.75 cc. of 0.1 N Mg(OH)₂ were added to one gram of casein, 42 per cent of the total alkaline earth element was present as cations, and the rest was combined with the protein as a negatively charged complex ion. Similarly, when 6.6 cc. of Ca(OH)₂ were added, 33 per cent was cationic; when

TABLE VII

Transport Numbers (Average Values at 25°).

B = cc. 0.1 N alkali per gm. of casein.

Q = electrochemical equivalent per millifaraday.

 $K = Q \times B$.

Time	Casein	B approxi- mately	pН	Q	K	$T_{ m casein}$	$T_{ m cation}$
		(a) (Casein + I	$Mg(OH)_2$			
Hr.	Per Cent	cc.					
3.5	1.85 - 2.25	6.75	7.0	1.20	8.1	0.84	0.26
3	2.27	7.5	7.7	0.90	6.8	0.82	0.31
2.5 - 3.5	1.85 - 2.55	9.5	9.3	0.74	7.1	0.74	0.40
3	2.28	10.45	9.8	0.66	6.9	0.71	0.27
3.5	1.7 - 2.2	10.9	10.0	0.56	6.2*	0.79	0.20
				Average	=7.2		
		(b)	Casein+	·Ca(OH) ₂			
3.5-4.0	1.94-2.57	6.6	7.2	1.15	7.6	1.00	(-0.12)
3.25	1.77	7.7	7.7	0.99	7.6	1.05	(-0.18)
3.5-4.0	1.6 - 2.1	9.5	9.4	0.85	8.1	0.78	1
3.25	1.84	11.0	10.3	0.63	7.0	0.64	
				Average	$=\frac{-}{7.5}$		
		(c)	Casein+	Sr(OH) ₂			
3.5-4	2.13	6.25	6.8	1.31	8.2	0.73	0.24
3.0-4.25	2.13	8.0	7.6	1.02	8.2	0.75	0.25
3.25	2.2	9.0	8.7	0.88	7.9	0.70	0.23
2.5	2.25	10.5	10.0	0.71	7.4	0.63	0.43
							1
				Average	e = 7.9		
		(d)	Casein+1	Ba(OH) ₂			
3.75	1.85	7.4	7.3	0.94	7.0	1.24	(-0.07)
4	1.85	8.0	8.0	0.80	6.4	1.06	(-0.09)
4	1.9	9.0	9.5	0.80	7.2	0.84	0.07
3.25	1.81	10.5	9.9	0.70	7.3	0.77	0.31
$\frac{3.25}{2.3}$	1.82	13.55	10.7	0.42	5.7*	0.54	0.42
				Average	 -7.0		

^{*} Not used in obtaining average. (Greenberg, D. M., and Schmidt, C. L. A., J. Gen. Physiol., 8, 271 (1926).)

6.25 cc. of Sr(OH)₂ were added, 46 per cent was present as cations; and when 7.4 cc. of Ba(OH)₂ were added, 26 per cent of the barium was present as cations.

The formation of complex metallic-containing protein ions is of great biological significance. The presence of a non-diffusible calcium fraction in blood serum is due to complex negative ions of

Table VIII

Transport of Electrodialyzed Serum in a Single Alkali Solution

Serum sample	Serum proteins	Alkali per gm. of protein	pН	$T_{ m serum}$ -	$T_{ m cation}$
		Alkali, Na	ıОН		
	per cent	meq.			
S ₂	2.0	0.28			0.60
	2.0	0.28			0.57
	1.2	0.24	7.9	0.25	100
	1.2	0.24	7.9	0.25	
S_3	3.5	0.23	7.9	0.18	0.70
	3.5	0.23	7.9	0.19	0.61
S_4	3.3	0.25	8.2	0.23	
	3.3	0.25	8.2	0.21	
Average				0.22	0.62
		Alkali, C	a(OH) ₂		
S ₂	3.2	0.26	8.2	0.32	
	3.2	0.26	8.2	0.32	
	3.2	0.27	8.8	0.27	0.48
	3.2	0.27	8.8	0.27	0.55
S ₂	4.4	0.22	8.0	0.21	0.75
	4.4	0.22	8.0	0.20	(0.95)?
Average				0.27	0.60

(Greenberg, D. M., J. Biol. Chem., 79, 177 (1928).)

the type (11), $B_n P Ca_m = nB^+ + P Ca_m^{-n}$, where B represents the monovalent bases and P the proteins of the serum. Experimental evidence which is favorable to this idea was obtained by Greenberg (12) from transport experiments in solutions of electrodialyzed blood serum to which sodium or calcium hydroxide or a mixture of the two was added (see Tables VIII and IX). The average transport number of the sodium cation was found to be 0.62, and that of the calcium cation 0.60. From the mobilities of the sodium and

calcium ions which, at 25°, are 50 and 60 mhos, respectively, it would be expected that the transport numbers of these ions should be in this ratio, provided only simple ions are present. Evidently the low transport number of the calcium cation is due to the pres-

Table IX

Electrical Transference of Electrodialyzed Serum in Mixed Solutions of Sodium and

Calcium Hydroxide

-		Ъ								T .	~
		Base a	aaaea		*			$T_{rac{1}{2} ext{Ca}}$ +	+	Loss of from an	
			gm.		I)2*					TIOIII air	
ple	Serum proteins	gm	90		Ratio of Ca(OH) Titratable base		*				
Serum sample	rot	er	Ca(OH) ₂ per protein		C C	$T_{ m serum}$ proteins	$T_{ m titratable\ base}$	_	pa		pa
ığ.	d u	VaOH p protein	Sa(OH) protein		of	pro	able	Obtained	Calculated	_	Calculated
ran	run	NaOH protei	ot c		itr	ra m	trat	tai	leu	oun	leu
Se	Se	Ng d	ಬ್ಬ್ ಟ್ಟ್	μd	Ra 1	$T_{\rm se}$	$T_{ m ti}$	op	ပ္မ	Found	Ca
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
	per	meq.	meq.		per					mg.	mg.
	cent		a de milion		cent						
S_3	4.00	0.195	0.055	8.0	22	0.23		(-0.08)	0.15	(-1.15)	2.2
~	4.00	0.195	0.055	8.0	22	0.24	1	(-0.08)	0.15	(-0.65)	1.3
S_4	3.45	0.226	0.07	9.3	24	0.31	0.52	(-0.09)	0.16	(-0.65)	1.2
	3.45	0.226	0.07	9.3	24	0.32	0.51	(-0.11)	0.16	(-0.50)	0.8
	$3.49 \\ 3.49$	0.135	$0.128 \\ 0.128$	8.8	49 49	0.29	0.49	(-0.02)	0.33	(-0.05)	2.0
	3.43	0.133	0.128	8.8	19	0.30	0.55	0.0	0.33	0.0	1.3
	3.43	0.204	0.048	8.7 8.7	19	$0.28 \\ 0.27$		0.0	$0.13 \\ 0.13$	$0.0 \\ (-0.1)$	$\begin{array}{ c c } 1.1 \\ 0.7 \end{array}$
	3.67	0.174	0.09	8.6	34	0.27	0.62	0.01	0.13	0.0	1.6
	3.67	0.174	0.09	8.6	34	0.31	0.49	0.03	0.23	0.3	1.0
S_5	4.22	0.168	0.079	8.3	32	0.27	0.10	0.0	0.21	0.0	1.5
	4.22	0.168	0.079	8.3	32	0.25		(-0.02)	0.21	(-0.1)	1.0
	3.70	0.199	0.088	8.4	30	0.29		0.03	0.20	0.15	1.9
	3.70	0.199	0.088	8.4	30	0.30		0.05	0.20	0.15	1.2
	4.26	0.184	0.048		21	0.23	0.63	0.04	0.14	0.3	1.1
	4.26	0.184	0.048		21	0.21	0.66	0.04	0.14	0.2	0.8
Av	erage					0.28	0.56				

^{*} Titratable base represents the sum of sodium and calcium. (Greenberg, D. M., J. Biol. Chem., 79, 177 (1928).)

ence of complex calcium-containing protein anions. Similarly, when the ratio 60/50 was used to calculate the transport number of the calcium cation when both calcium and sodium ions were present in the protein solution, the calculated value, based on the assumption that only simple calcium ions were present in the solutions, deviated widely from the experimental values.

5. TRANSPORT NUMBERS OF AMINO ACIDS

Transport experiments have been carried out on the dicarboxylic amino acids by Miyamoto and Schmidt (13). Their object was to throw some light on the nature of the groups present in the protein molecule which are responsible for the formation of complex ions with the alkaline earth elements. To prevent changes in acidity in the cathode compartment, the platinum wire was covered with aspartic or glutamic acid crystals, and the whole was enclosed in a

Table X

Transference Numbers of Sodium and Calcium Caseinate and of Sodium and Calcium

Dephosphorized Caseinate at 30°

	Q	В	QB = K	$T_{ m case in}$ —	$T_{ m cation}$	$T_{ ext{casein}-} + T_{ ext{cation}}$	i
							per cent
Sodium case-	1.37	6.52	8.95	0.499	0.542	1.041	
inate	1.09	8.55	9.33	0.462	0.534	0.996	
	1.02	9.05	9.23	0.454	0.548	1.002	
*	1.73	5.6	9.68	0.453	0.561	1.014	
*	1.34	7.0	9.40	0.455	0.540	0.995	
*	1.04	8.25	8.60	0.430			
Sodium dephos-	1.39	6.47	9.00	0.389	0.595	0.984	
phorized	1.17	6.50	7.61	0.428	0.586	1.014	
caseinate	1.15	6.83	7.89	0.383	0.575	0.958	
	1.24	7.30	9.05	0.388	0.700	1.088	
	1.12	7.90	8.85	0.396	0.625	1.021	
	0.945	8.85	8.36	0.424	0.593	1.017	
Calcium case-	0.411	12.23	5.03	0.953	0.331	1.284	48.7
inate	0.331	12.95	4.28	1.185	0.338	1.523	49.3
	0.300	13.20	3.96	0.968	0.194	1.162	40.5
	0.2195	14.70	3.23	1.295	0.343	1.638	49.7
	0.196	14.75	2.90	1.070	0.278	1.348	45.4
	0.197	15.40	3.02	1.200	0.199	1.399	40.8
Calcium de-	0.962	7.49	7.20	0.542	0.479	1.021	62.8
phosphorized	0.972	8.48	8.24	0.564	0.533	1.097	69.8
caseinate	0.723	9.54	6.90	0.608	0.416	1.024	55.9
	0.614	11.80	7.24	0.676	0.431	1.107	57.9
	0.666	10.85	7.22	0.580	0.408	0.988	55.1
	0.614	12.35	7.57	0.686	0.395	1.081	54.0
	0.614	12.35	7.58	0.657	0.441	1.098	58.2
	0.481	13.43	6.47	0.630	0.430	1.060	57.3
	0.531	14.10	7.46	0.700	0.422	1.122	56.6

 $B={\rm cc.}$ of 0.1 N alkali per gm. of casein; $Q={\rm electrochemical}$ equivalent per millifaraday; $i={\rm fraction}$ of metallic element which exists as ions; $T={\rm transport}$ number.

(Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

^{*} These data are taken from Greenberg and Schmidt (10).

Table XI
Transference Numbers of Amino Acid Solutions at 25°

	$T_{ m amino\ acid}$	$T_{ m cation}$	$T_{ ext{amino acid}} - \ + T_{ ext{cation}}$	
Monosodium aspartate	0.351			
	0.349			
	0.350			
	0.357			
	0.354			
	0.352			
Monosodium glutamate	0.319			
	0.322			
	0.313			
	0.325			
	0.324			
	0.315			
Barium diaspartate	0.307	0.676	0.983	
	0.297	0.690	0.987	
	0.305	0.695	1.000	
	0.298	0.705	1.003	
	0.301	0.704	1.005	
	0.301			
Barium diglutamate	0.279	0.739	1.018	
	0.289	0.709	0.998	
	0.278	0.710	0.988	
	0.275	0.725	1.000	
	0.277	0.717	0.994	
	0.279	0.718	0.997	
	0.294	0.711	1.005	
Calcium diaspartate	0.336	0.661	0.997	
	0.322	0.685	1.007	
	0.319	0.678	0.997	
	0.329	0.682	1.011	
	0.311	0.691	1.002	
	0.306	0.683	0.989	
	0.321	0.698	1.019	
	0.305	0.692	0.997	
Calcium diglutamate	0.306	0.702	1.008	
[14] 회장 [14] [2.1 (17.15 - 17.15)	0.307	0.681	0.988	
	0.291	0.701	0.992	
	0.306	0.702	1.008	
	0.294	0.720	1.014	
	0.300	0.702	1.002	
	0.296	0.708	1.004	

(Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

collodion or filter paper bag. Since these acids are fairly insoluble, the acidity is not materially altered. The platinum anode was similarly covered with barium or calcium carbonate and placed in

a bag. Stirring of the solution was prevented by placing the bag in the top of the compartment. In carrying out transport experiments in solutions of the sodium salts of these amino acids, metallic cadmium was used for the anode in order to prevent changes in acidity. The difficulty arose in that, besides sodium ions, cadmium ions also migrated to the cathode, which prevented the calculation of the transport numbers of the cations. Conductivity experiments were also carried out.

The average transport values of the sodium, calcium, and barium salts of aspartic and of glutamic acid and the sodium and calcium salts of casein obtained by Miyamoto and Schmidt are given in Tables X and XI. The data show no evidence of the presence of complex ions in solutions of the sodium, barium, and calcium salts, respectively, of glutamic and aspartic acid. Solutions of sodium caseinate yielded normal transport values, while calcium caseinate solutions gave transport numbers which were greater than unity. Evidently the seat of complex ion formation cannot be attributed directly to the free carboxyl groups of the protein molecule. It is not probable that the hydroxyl group of β -hydroxyglutamic acid is concerned with complex ion formation since lactic acid does not yield complex ions with calcium (14). No groups similar to those which occur in citric acid, a compound which forms complex ions with calcium, appear to be present in the protein molecule.

Since, within the range of acidity at which transport experiments have been carried out in solutions of calcium or barium proteinates, the alkali earth element is largely, if not entirely, bound to the free carboxyl groups, it appears that these groups are nevertheless the seat of complex ion formation. Due to the large content of free carboxyl groups, casein can be considered as being a polybasic acid. It is conceivable that the alkaline earth element which is bound to certain of the free carboxyl groups is more easily ionized than that which is bound to the remainder. The net result is step dissociation of the type which takes place in polybasic acids. If this is true, the non-ionized calcium which is bound to the carboxyl groups will, in transport experiments, be carried by the current in a direction opposite to the path of migration of the dissociated metallic ions. Evidence for the formation of complex

¹ J. C. Abels (J. Amer. Chem. Soc., 58, 2609 (1936)) considers that the functional group in proteins which binds calcium is the hydroxyl group. The basis for the conclusion is that acetylated egg albumin shows very little calcium-binding power while native egg albumin does so.

barium-containing protein ions is given in the section of this chapter dealing with amalgam electrodes. Dephosphorized casein forms about 10 per cent less complex ions with calcium than does

Table XII

Comparison of Mobilities of Anions from Data of Conductivities and of

Transference Numbers*

Compound	$\Lambda_{ m 0~compound}$	$\Lambda_{0 ext{ anion}}$ (c)	$\Lambda_{0 ext{ anion (t)}}$	$\Lambda_{0 ext{ anion (o)}}$ minus $\Lambda_{0 ext{ anion (t)}}$
Sodium caseinate	100.00	44.20	45.50	-1.30
Calcium caseinate	85.20	19.10	+	
Sodium dephosphorized caseinate	93.00	37.20	37.00	0.20
Calcium dephosphorized caseinate	92.50	26.40	11.00	15.40
Monosodium aspartate	78.27	27.75	27.60	0.15
Barium diaspartate	91.96	27.75	27.50	0.25
Calcium diaspartate	86.57	27.75	27.70	0.05
Monosodium glutamate	75.62	25.10	25.00	0.10
Barium diglutamate	89.31	25.10	25.00	0.10
Calcium diglutamate	83.92	25.10	25.20	-0.10
Aspartic acid	377.74	27.75		- 1 T - 114
Glutamic acid	375.09	25.10		

^{*} The values for the casein and the dephosphorized casein compounds are at 30°. The values for the amino acid compounds are at 25°. $\Lambda_{0 \text{ anion (c)}}$ is calculated from conductivity measurements; $\Lambda_{0 \text{ anion (t)}}$ is calculated from transference experiments. The following values were used for the cation mobilities.

	At 25°	At 30°
$\Lambda_{0 \text{ Na}}^+$ $\Lambda_{0 \frac{1}{2} \text{Ca}}^+$	50.52 58.82	55.80 66.10
$\Lambda_{0\frac{1}{2}Ba}^{++}$ Λ_{0H}^{+}	64.21 349.99	374.80

[†] Since, due to the presence of complex ions in the solution, the transference number of calcium caseinate is greater than unity, the calculated value for $\Lambda_{0 \text{ anion (t)}}$ is negative. This is an irrational value.

(Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

casein. Evidently some of the calcium is bound to the phosphoric acid of casein and is carried in a non-dissociated form.

A priori, according to this hypothesis, there is no reason why the alkali metals should not likewise yield complex ions with proteins. It is quite possible that such ions may be formed. The number must, however, be less than the limits of error (10 per cent) in transport experiments. The transport data do not lend support to the conclusions of Greene and Power (15) who have reported, from

TABLE XIII

Electrical Transference of Proteins in Formic Acid

Temperature, 25°

a. Casein

Experi- ment No.	Casein concen- tration	Coulom- eter current	Compart- ment	Volume of com- partment	Nitrogen titration change of compart- ment	Casein change in compartment		$T_{ m casein}^+$
1	gm. per liter 30.0	milli- equivalents 0.455 0.455	Anode Cathode	ml. 17.5 17.0	milli- equivalents 0.443 0.430	mg. -42.4 +41.6	milli- equivalents -0.043 +0.042	0.095 0.092
2	30.0	1.065 1.065	Anode Cathode	19.6 17.5	0.995 1.025	-95.5 +97.5	-0.097 +0.099	0.090 0.093
Aver:	Ta							0.093 5.5

b. Edestin

Ex- peri- ment No.	Edestin concen- tration	Coulom- eter current	Compart- ment	Volume of com- partment	Nitrogen titration change of com- partment	Edestin change in com- partment	Edestin change N = 640	$T_{ m edes-} \ m _{tin}^+$	Edestin change N =750	$T_{ m edes-} \atop m tin^+$
	gm. per liter	milli- equivalents		ml.	milli- equivalents	mg.	milli- equivalents		milli- equivalents	
3	27.0	1.360	Anode	20.1	1.68	-121	-0.189	0.14	-0.161	0.12
4	28.5	0.775	Anode	18.8	1.00	-76	-0.1175	0.15	-0.1015	0.135
		0.775	Cathode	18.6	0.99	+75	+0.117	0.15	+0.1000	0.13
	erage destin ⁺							0.15 9.0		0.13 7.7

c. Gelatin

Experi- ment No.	Gelatin concen- tration	Coulom- eter current	Compart- ment	Volume of com- partment	Nitrogen titration change of compart- ment	Gelatin change in compartment N=950		$T_{ m gelatin}^+$
5	gm. per liter 30 30	milli- equivalents 1.27 1.27	Anode Cathode	ml. 17.8 16.4	milli- equivalents 0.554 0.562	mg153 +144	milli- equivalents -0.160 +0.150	0.125 0.12
Average Aogelati								0.12 7.2

(Greenberg, D. M., and Larson, C. E., J. Phys. Chem., 39, 665 (1935).)

diffusion experiments, that approximately 11 per cent of sodium and 24 per cent of potassium are bound to certain proteins in a non-diffusible form.

A comparison of the mobilities of casein and the several amino acid ions is given in Table XII. The values for the amino acid ions are considerably less than similar values for the mobility of the casein ion or of the acetate ion.

6. TRANSPORT NUMBERS OF PROTEINS IN FORMIC ACID

Greenberg and Larson (16) have determined the transport numbers of several proteins which were dissolved in anhydrous formic acid. Table XIII summarizes their data. The Λ_0 values of the proteins were calculated by using the value of 51.6 for the equivalent conductivity of the formate ion. Compared with the values in aqueous solution, the protein ion mobilities are low, but they are in line with the figures for the mobility of the alkali and alkaline earth elements in formic acid as found by Schlesinger and his associates (17).

7. TRANSPORT NUMBERS FROM DIFFUSION POTENTIALS

In place of the Hittorf method, Ferguson and co-workers (18) and Prideaux (19) have employed the concentration cell method for the determination of transference numbers in uni-univalent and uni-bivalent electrolytes. The determination involves the measurement of the potentials of (a) a concentration cell without diffusion, (b) a concentration cell with diffusion and reversible with respect to the cation, and (c) a concentration cell with diffusion and reversible with respect to the anion. In the case of sulfuric acid, the cells are:

- (a) $Pt_{H} \mid 0.1 \text{ M } H_{2}SO_{4}, Hg_{2}SO_{4} \mid Hg \mid Hg_{2}SO_{4}, 0.01 \text{ M } H_{2}SO_{4} \mid Pt_{H}$
- (b) $Pt_H \mid H_2SO_4 c_1 \mid H_2SO_4 c_2 \mid Pt_H$
- (c) Hg \mid Hg₂SO₄, H₂SO₄ $c_1 \mid$ H₂SO₄ c_2 Hg₂SO₄ \mid Hg

The potential of cell (a) is given by the equation,

$$E = \frac{3}{2} \frac{RT}{F} \ln \frac{c_1}{c_2} \tag{11}$$

The value E is obtained experimentally from the difference between the potentials of the cells.

 $Pt_{H} \left| \ 0.1 \ M \ H_{2}SO_{4}, \ Hg_{2}SO_{4} \right| \ Hg \ \text{ and } \ Pt_{H} \left| \ 0.01 \ M \ H_{2}SO_{4}, \ Hg_{2}SO_{4} \right| \ Hg$

The total potential of cell (b) consists of the algebraic sum of the two electrode potentials and the potential at the boundary of the

solutions. Assuming that sulfuric acid is completely dissociated, the algebraic sum of the electrode potentials is given by the Nernst equation,

 $E_1 = \frac{RT}{F} \ln \frac{c_1}{c_2} \tag{12}$

The potential at the liquid boundary is given by

$$E_{\rm B} = \frac{2U_c - U_a}{2(U_c + U_a)} \frac{RT}{F} \ln \frac{c_1}{c_2}$$
 (13)

where U_c and U_a are, respectively, the mobility of the cation and anion. The hydrogen electrode in the concentrated solution is positive with respect to the hydrogen electrode in the dilute solution. Since, at the boundary of the solutions, the sulfuric acid diffuses from the concentrated to the dilute side, and since the hydrogen ion moves faster than the sulfate ion, the dilute side is charged positively with respect to the concentrated side which means that the potential which is developed at the boundary opposes that of the hydrogen electrodes. The total potential of the hydrogen concentration cell is expressed by the equation,

$$E_1 - E_B = E_3 = \frac{RT}{F} \ln \frac{c_1}{c_2} - \frac{2U_c - U_a}{2(U_c + U_a)} \frac{RT}{F} \ln \frac{c_1}{c_2} = \frac{3}{2} \frac{U_a}{U_a + U_c} \frac{RT}{F} \ln \frac{c_1}{c_2}$$

Substituting the transport number N_a of the anion for U_a/U_a+U_c , the above equation becomes

$$E_{\rm H} = \frac{3}{2} N_a \frac{RT}{F} \ln \frac{c_1}{c_2} \tag{14}$$

The total potential of cell (c) consists of the algebraic sum of the two electrode potentials and the potential at the boundary of the solutions. The algebraic sum of the electrode potentials is expressed by

$$E_4 = \frac{RT}{2F} \ln \frac{c_1}{c_2} \tag{15}$$

The boundary potential is the same as in the hydrogen concentration cell. It is also in the same direction. The algebraic sum of the sulfate electrode potentials is likewise in this direction. The total potential of the sulfate concentration cell is expressed by the equation,

$$E_4 + E_B = E_{SO_4} = \frac{RT}{2F} \ln \frac{c_1}{c_2} + \frac{2U_c - U_a}{2(U_c + U_a)} \frac{RT}{F} \ln \frac{c_1}{c_2} = \frac{3U_c}{2(U_c + U_a)} \frac{RT}{F} \ln \frac{c_1}{c_2}$$

Substituting the transport number, N_c of the cation for the expression U_c/U_a+U_c , the above equation becomes

$$E_{SO_4} = \frac{3}{2} N_c \frac{RT}{F} \ln \frac{c_1}{c_2}$$
 (16)

On dividing equation (16) by equation (11), $N_c = E_{\text{SO}_4}/E$. Similarly, $N_a = E_{\text{H}}/E$, since $N_a + N_c = 1$. Therefore, $E_{\text{SO}_4}/E + E_{\text{H}}/E = 1$, and

$$E_{SO_4} + E_H = E \tag{17}$$

From equation (17) it is evident that the value which is obtained from potentials $E_{\rm SO_4} + E_{\rm H}$ is the same as that obtained by the difference of potentials $E_{0.01}$ and $E_{0.1}$ (cell a, p. 655). In order to obtain the potential $E_{\rm SO_4}$, the boundary potential is added to the electrode potentials, while for the total potential, $E_{\rm H}$, it is subtracted. Combining the expressions

$$E_{\rm H} = \frac{RT}{F} \ln \frac{c_1}{c_2} - \frac{(2 - 3N_a)}{2} \frac{RT}{F} \ln \frac{c_1}{c_2}$$

$$E_{\rm SO_4} = \frac{RT}{2F} \ln \frac{c_1}{c_2} + \frac{(2 - 3N_a)}{2} \frac{RT}{F} \ln \frac{c_1}{c_2}$$

we obtain

$$2E_{SO_4} = \frac{RT}{F} \ln \frac{c_1}{c_2} + \frac{2(2-3N_a)}{2} \frac{RT}{F} \ln \frac{c_1}{c_2}$$

or

$$\frac{2E_{SO_4} - E_H}{3} = \frac{(2 - 3N_a)}{2} \frac{RT}{F} \ln \frac{c_1}{c_2}$$
 (18)

The value for the boundary potential may be obtained by substituting the measured potentials $E_{\rm SO_4}$ and $E_{\rm H}$ in equation (18).

Considerable numbers of experiments, which are based on the above method, have been reported on the mode of combination of proteins with acids or bases. We may illustrate the application of the diffusion method to the study of the mobility of the gelatin ion in solutions of sodium gelatinate. The following cells were used:

 E_{a}

- (a) H_2 Pt | NaOH c | NaOH c+x gel | H_2 Pt
- (b) H₂ Pt | NaOH c | | KCl sat. | | NaOH c+x gel | H₂ Pt
- (c) Na_y Hg | NaOH c | | KCl sat. | | NaOH c gel x | Na_y Hg.

The difference between (a) and (b) gives the diffusion potential E_d . Sodium hydroxide solutions were treated with gelatin until there was no change in potential, i.e., the activity of hydroxyl ions in the gelatin solution was constant. Then

$$E = \frac{RT}{F} \log \frac{a_{\text{(OH)}_2}}{a_{\text{(OH)}_1}} = K \tag{19}$$

Cell (c) gave potentials which hardly change with change of x, showing that the value of $a_{\rm Na}$ was hardly affected by the substitution of gelatinate by hydroxyl ions. When the x/E curves of (a) and (b) became parallel, indicating that the diffusion potentials were constant, it was assumed that the alkali was saturated with gelatin.

On the assumption that the gelatin ion is univalent (the migration is considered per equivalent of negatively charged gelatin) the diffusion potential was calculated from the equation,

$$E_d = \frac{RT}{F} \ln \frac{U + V_1}{U + V_2} \tag{20}$$

where V_1 is the mobility of the gelatin anion, V_2 that of the hydroxyl ion, and U that of the sodium ion. In order to calculate values for E_d , it was necessary to assume a value for the mobility of the gelatin ion. A good correlation between the observed and the calculated results was obtained by Ferguson and Schluchter (18) by assuming that the value of the mobility of the gelatin ion is 20. Prideaux (19) has reported a like value. The method of determining mobility of protein ions as given above is probably less accurate and more laborious than other methods which have been described in this chapter. The general conclusion which has been drawn from these experiments is that the diffusion potentials which are given by solutions of gelatin salts can only be understood on the basis that the protein salt is highly ionized. The equivalent weight of gelatin in solutions of gelatin chloride was calculated to be 1090.

8. CONDUCTIVITY OF PROTEINS AND AMINO ACIDS

Being electrolytes, solutions of the salts of proteins and amino acids are fairly good conductors of the electric current. The conductivities of the free amino acids depend on their degree of ionization, this being least in the case of the monoaminomonocarboxylic acids. Conductivity measurements on solutions of the salts of amino acids and proteins can be carried out only in regions

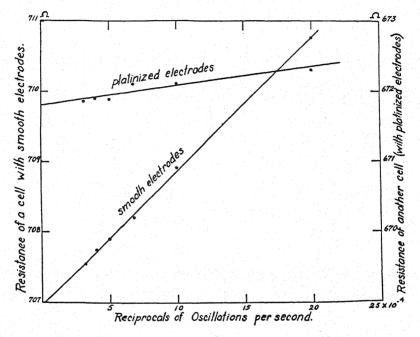


Fig. 4. Resistance measurements of a gelatin solution at varying frequency in a cell with smooth and in a cell with platinized electrodes (19.4°) .

(Gahl, R., and Greves, G. L., Univ. of Calif. Pub. Physiol., 5, 289 (1926).)

close to the neutral point since otherwise the excess number of H⁺ and OH⁻ will carry the larger fraction of the current.

Gahl and Greves (20) have made an extensive study of the technique of measuring the conductivity of protein solutions. They found that smooth platinum electrodes are best suited for measuring the conductivity of protein solutions. With smooth electrodes the conductivity of casein solutions remains stationary for considerable time, while with platinized electrodes it changes rather rapidly. On the other hand, measurements with smooth electrodes require much more elaborate apparatus and more time and care

than in the case of platinized electrodes. In addition to having to make measurements at several frequencies and having to extrapolate them to infinite frequency, since the electrical resistance is

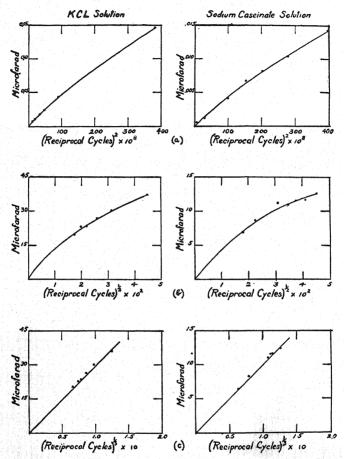


Fig. 5. Comparison between KCl solution and sodium caseinate solution when (a) Shunted capacity of a cell with smooth electrodes is plotted against squares of reciprocal cycles per second.

(b) Series capacity of a cell with smooth electrodes is plotted against square roots of reciprocal cycles per second.

(c). Series capacity of a cell with smooth electrodes is plotted against cube roots of reciprocal cycles per second.

(Gahl, R., and Greves, G. L., Univ. of Calif. Pub. Physiol., 5, 289 (1926).)

influenced by the frequency of the circuit, it is necessary, in order to obtain a sharp minimum, to adjust both resistance and capacity accurately; while with platinized electrodes a fair minimum is obtained without troubling about capacity. With smooth electrodes the current has to be perfectly harmonic due to the fact that different wave lengths require different capacity adjustments.

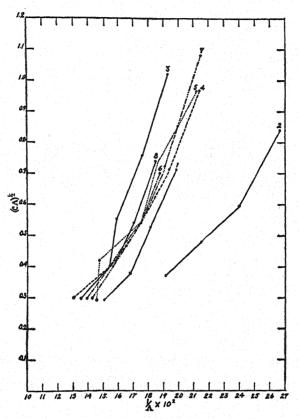


Fig. 6. Conductivity measurements of sodium caseinate by various authors, plotted according to Greenberg and Schmidt.

1. Pauli and Matula.	pH = 6.3
2. Robertson	pH = 6.3
3. Greenberg and Schmidt	pH = 6.5
4. Greenberg and Schmidt	pH = 6.5
5. Greenberg and Schmidt	pH = 6.5
6. Pauli and Matula	"slightly alkaline"
7. Laqueur and Sackur	"slightly alkaline"
& Greenherg and Schmidt	nH = 7.5

(Gahl, R., Greenberg, D. M., and Schmidt, C. L. A., Univ. Calif. Pub. Physiol., 5, 307 (1926).)

Extrapolation of conductivity measurements to an infinite series of cycles can be carried out by plotting the cell resistance against the reciprocals of oscillations per second. In general, a straight line is obtained when solutions of electrolytes are used (see Fig. 4).

Practically, this may be carried out by measuring the shunted resistance at two frequencies, one twice as high as the other. The

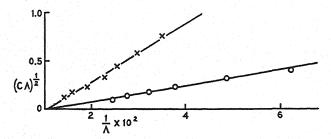


Fig. 7. Conductivity of calcium caseinate, \bigcirc , and of calcium dephosphorized caseinate, \times .

(Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

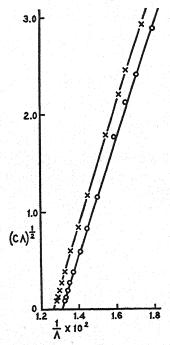


Fig. 8. Conductivity of sodium aspartate, \times , and of sodium glutamate, \bigcirc . (Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

difference in resistance between the two is determined. This difference is deducted from the lower resistance (the one corresponding to the higher frequency). The result is the resistance for infinite frequency. The variability of the resistance with frequency

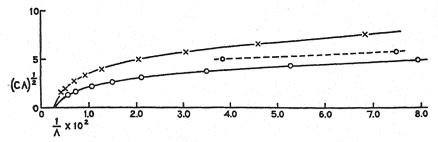


Fig. 9. Conductivity of aspartic acid, \times , and of glutamic acid, \bigcirc . The dotted line is the extension of the lower curve. The values of the ordinates are as given. The values of the abscissae must be increased by adding 4 to the values given, for the extension of the curve (dotted line).

(Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

is closely related to the existence of a polarization capacity at the surface of the electrodes. Insofar as variation in capacity is con-

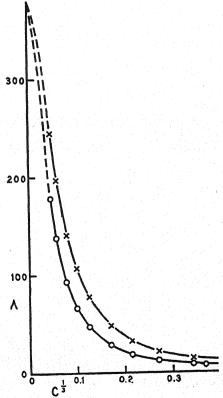


Fig. 10. Conductivity of aspartic acid, \times , and of glutamic acid, \bigcirc . (Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

cerned, casein solutions behave exactly like dilute solutions of potassium chloride. For both solutions the capacity, when represented by a shunted condenser, is nearly proportional to the squares of the reciprocal frequency, while, when represented by a condenser in series, it increases more slowly with increasing frequency than the square root, apparently as the cube root (see Fig. 5). Measurements of the conductivity of protein solutions

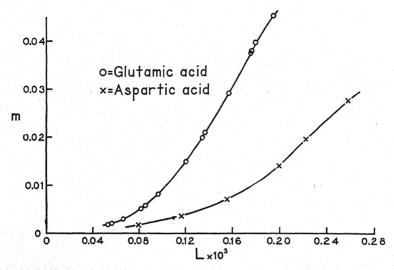


Fig. 11. Specific conductance of solutions of aspartic and glutamic acids at 0°. (Hoskins, W. M., Randall, M., and Schmidt, C. L. A., J. Biol. Chem., 88, 215 (1930).)

using smooth electrodes check, on an average, within a fraction of one per cent, both for platinized and smooth electrodes.

In extrapolating conductance data to infinite dilution, it is usually useful to employ some form of Storch's equation (21) which, for purposes of plotting, may be put into the form,

$$(C\Lambda)^n = f\left(\frac{1}{\Lambda}\right) \tag{21}$$

In the case of proteins and certain of the amino acid salts a straight line is obtained when $(C\Lambda)^{1/2}$ is plotted against $1/\Lambda$ where $\Lambda = \text{equivalent}$ conductivity, and C = concentration. This has been found to be true in the case of the alkali (22, 23) and alkaline earth caseinates and the sodium salts of aspartic and glutamic acid (13). The conductivity curves of the barium and calcium salts, respectively,

of these amino acids, deviate somewhat from a straight line when similarly plotted. Conductivity data which have been obtained by Gahl, Greenberg and Schmidt, and by Miyamoto and Schmidt are graphically represented in Figs. 6 to 10.2 The conductivities of aspartic and glutamic acid and their monosodium salts, plotted in different ways, are represented in Figs. 11 to 14.

Onsager (25) has formulated an expression which is based on the

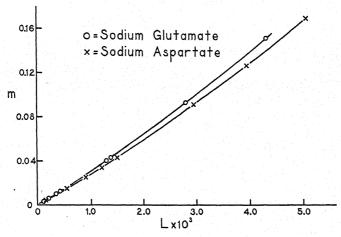


Fig. 12. Specific conductance of solutions of the monosodium salts of aspartic and glutamic acids at 0° .

(Hoskins, W. M., Randall, M., and Schmidt, C. L. A., J. Biol. Chem., 88, 215 (1930).)

Debye-Hückel (26) theory of the conductivity of strong electrolytes. In order to determine the status of the salts of amino acids and proteins in relation to the theory of strong electrolytes, Miyamoto and Schmidt (13) have plotted the conductivity of the sodium, calcium, and barium salts, respectively, of aspartic and glutamic acid and of casein according to the method of Onsager.

² According to Hoskins, Randall and Schmidt (24) conductivity data of amino acids may, for purposes of determining concentration in such work as freezing point measurements, be evaluated in terms of molality by plotting the logarithm of the molality against the logarithm of the conductance. The slope of the nearly straight line thus obtained is used in the equation,

$$\log m = a \log c + b,$$

where m = molality, c = conductance, b = a constant, and a = the slope of the curve. A second plot of $\log c$ against b is then made. This is very sensitive to changes in conductance. The graphs are used in the reverse order to estimate the concentration of amino acid.

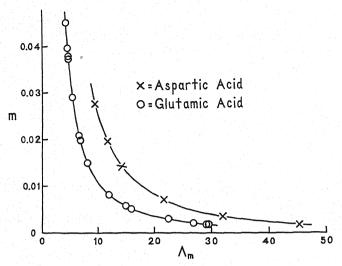


Fig. 13. Equivalent conductances of solutions of aspartic and glutamic acids at 0° .

(Hoskins, W. M., Randall, M., and Schmidt, C. L. A. J. Biol. Chem., 88, 215 (1930).)

The limiting formula for equivalent conductance may be written

$$\Lambda = \Lambda_0 - \alpha \sqrt{\Gamma} \tag{22}$$

where Λ = the molar conductivity and Γ = twice the ionic strength =

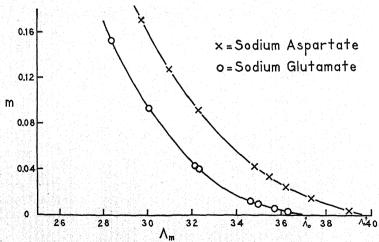


Fig. 14. Equivalent conductances of solutions of the monosodium salts of aspartic and glutamic acids.

(Hoskins, W. M., Randall, M., and Schmidt, C. L. A., J. Biol. Chem. 88, 215 (1930).)

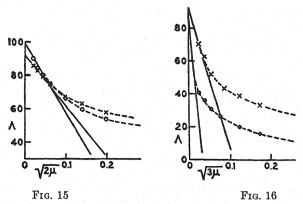


Fig. 15. Conductivity of sodium caseinate, \bigcirc , and of sodium dephosphorized caseinate, \times , plotted according to Onsager's equation.

Fig. 16. Conductivity of calcium caseinate, O, and of calcium dephosphorized caseinate, X, plotted according to Onsager's equation.

(Miyamoto, S., and Schmidt, C. L. A., Jour. Biol. Chem., 99, 335 (1933).)

 $(Z_1+Z_2\cdot\cdot\cdot)\mu$. The term $\mu=$ molar concentration, and Z_1 and $Z_2=$ valences of the anions and cations. The term $\alpha=$ a constant. At 25°, when the solvent is water, theoretical values for α are given by the equations,

$$\alpha = 35.7 + 0.159 \Lambda_0$$
 (for uni-univalent electrolytes)

$$\alpha = 53.55 + 1.084 \frac{q}{1 + \sqrt{q}} \Lambda_0$$
 (for uni-bivalent electrolytes)

where $q = \Lambda_0/(\Lambda_0 + l_1)$ and l_1 is the mobility of the univalent ion. When $\sqrt{\Gamma}$ is plotted against Λ , the curve will be a straight line.

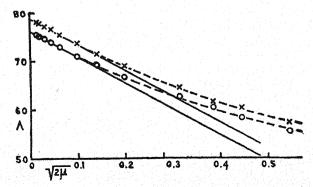


Fig. 17. Conductivity of sodium aspartate, X, and of sodium glutamate, O, plotted according to Onsager's equation.

(Miyamoto, S., and Schmidt, C. L. A., Jour. Biol. Chem., 99, 335 (1933).)

However, Onsager found a deviation from a straight line for a number of strong electrolytes in that the curvature of the curve was upward.

By plotting Λ against $\sqrt{n\mu}$, where μ = molecular concentration and n is a number (2 or 3), the curves which are shown in Figs. 15 to 17 were obtained. They have the same general shape as those which were found for strong electrolytes by Onsager. This may be taken as an indication that the behavior of solutions of these

Table XIV

Experimental and Calculated Values for α in Onsager's Equation

Salts	Λ_0	αexperimental	$lpha_{ ext{theoretical}}$	$lpha_{ ext{experimental}}$ minus $lpha_{ ext{theoretical}}$
Sodium chloride	108.89	54.69	53.00	+1.70
Monosodium glutamate	75.62	50.80	47.72	+3.08
Monosodium aspartate	78.27	51.70	48.15	+3.55
Barium nitrate	116.95	92.80	86.90	+5.90
Barium diaspartate	91.96	93.30	94.45	-1.15
Barium diglutamate	89.31	95.30	93.45	+1.65
Calcium chloride	116.69	88.00	86.10	+1.90
Calcium diaspartate	86.57	90.80	91.25	-0.45
Calcium diglutamate	83.92	89.80	90.75	-0.95
Sodium caseinate	100.00	425.0	51.6	+374.4
Sodium dephosphorized caseinate	93.00	290.0	50.5	+239.5
Calcium caseinate	85.20	2750.0	87.0	+2663.0
Calcium dephosphorized caseinate	92.50	870.0	92.3	+777.7

 $\Lambda = \Lambda_0 - \alpha \sqrt{\Gamma}$ (Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

amino acids and of casein does not differ materially from that of the strong electrolytes.

The experimental and calculated values for α in Onsager's equation are given in Table XIV. It is to be noted that the values for α of the monosodium salts of aspartic acid and of glutamic acid are typical of those for a uni-univalent electrolyte such as sodium or potassium chloride, while those of the barium and calcium salts of these amino acids fall well within the limits which are obtained with bi-univalent electrolytes such as barium nitrate or calcium chloride. In calculating values for α in the case of casein the assumption was made that this protein behaves as a univalent ion. The reason for the large difference between the calculated and the experimental values for α in the case of casein is not clear. It may perhaps be due to extensive aggregation of the casein molecules.

9. OSTWALD'S DILUTION LAW

In 1904 Walker (27) showed that the relations between the acidity of a solution of an amphoteric electrolyte and the dissociation constants can be expressed by the equation,

$$(H^{+})^{2} = \frac{K_{w} + k_{a}u}{1 + k_{b}u/K_{w}}$$
 (23)

in which k_a and k_b are the classical acid and basic dissociation constants, respectively, of the amphoteric substance, u is the concentration of the undissociated molecule, and K_w is the dissociation constant of water. Walker stated "when k_b/K_w and u have finite values it is obvious that amphoteric electrolytes cannot strictly obey Ostwald's dilution law. If, however, either k_b/K_w or u is very small, Ostwald's dilution law is approximately followed, for then the values of H+ from the simple and amphoteric formulae become nearly equal . . . The smaller the basic dissociation constant, then, and the greater the dilution, the more likely is the amphoteric electrolyte to follow the dilution law characteristic of simple acids and bases."

Now Ostwald's dilution law is derived from the following considerations (28a). On the assumption of constant ionic mobility, Λ/Λ_0 gives the degree of dissociation at each concentration and $m\Lambda/\Lambda_0$ gives the molality of each ion, while $m(1-\Lambda/\Lambda_0)$ gives the molality of the undissociated substance. We may consider that K_{Λ} is defined by the equation,

$$K_{\Lambda} = \frac{m(\Lambda/\Lambda_0)^2}{(1 - \Lambda/\Lambda_0)} \tag{24}$$

If in a reaction of the type $XY = X^+ + Y^-$ the assumption is made that the activity of each substance is equal to its molality, which, however, is only approximately true, then $K_{\Lambda} = K$. This expresses Ostwald's dilution law. In equation (24) conductivity values which represent activity values have been substituted in the mass law equation, $K = (A)^2/HA$, which expresses the dissociation of a weak acid.

Walker showed that, in the case of amino benzoic acid, Ostwald's dilution law is only approximately followed. Acetic acid obeys this law even in fairly high (0.13 M) concentration. On the other hand, cacodylic acid, which has a smaller dissociation constant than acetic acid, obeys the dilution law only approximately (29).

Table XV
Conductivity Data of Aspartic Acid, Glutamic Acid, and Glycine at 25°

	m	$L \times 10^5$	Λ	α*	$K_{\Lambda} \times 10^4$
Aspartic acid	0.0000		377.74	1.000	1.50
	0.0001	2.450	245.00	0.649	1.19
	0.0002	3.940	197.00	0.522	1.14
	0.0005	7.048	140.96	0.373	1.12
	0.001	10.67	106.70	0.283	1.12
	0.002	15.61	78.05	0.207	1.08
	0.005	24.33	48.66	0.129	0.955
	0.01	32.69	32.69	0.0867	0.823
	0.02	43.41	21.70	0.0576	0.704
	0.04	58.38	14.60	0.0387	0.623
					K _A ×10⁵
Glutamic acid	0.0000		375.09	1.000	5.62
	0.0001	1.782	178.20	0.475	4.31
	0.0002	2.760	138.00	0.368	4.28
	0.0005	4.651	93.12	0.248	4.13
	0.001	6.641	66.41	0.181	3.99
	0.002	9.461	47.31	0.126	3.63
	0.005	14.30	28.60	0.0763	3.39
	0.01	18.90	18.90	0.0504	2.68
	0.02	25.15	12.58	0.0335	2.35
	0.04	34.74	8.69	0.0232	2.22
	0.05	39.09	7.82	0.0209	2.21
		L×106		α×104	K _A ×10 ¹⁰
Glycine	0.00		380.0000†		2.54
	0.01	1.183	0.1183	3.12	9.75
	0.02	2.124	0.1062	2.80	15.70
	0.05	3.818	0.07636	2.00	20.00
	0.1	6.978	0.06978	1.87	35.00
	0.2	13.62	0.06810	1.79	64.00
	0.5	26.81	0.05362	1.41	99.50
	1.0	50.88	0.05088	1.34	179.50

The dissociation constant, K, of the amino acids given in Table XV has been taken from the table of Miyamoto and Schmidt. However, the dissociation constant of aspartic acid given in their table is the one calculated from the data of (ΔH) at 25°. We, therefore, have chosen the value of Lundén for aspartic acid.

* α (classical degree of dissociation) = Λ/Λ_0 .

† The mobility of the glycine ion is assumed to have the value 30.

(Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 99, 335 (1933).)

Robertson (30) showed that solutions of certain proteins in various acids and alkalies followed Ostwald's law quite well. He was forced to make the assumption in his calculations that the equivalent

concentration of the acid or base which is neutralized by the protein bears a constant proportion to the true equivalent concentration of the protein salt.

Miyamoto and Schmidt (13, 31) attempted to apply Ostwald's dilution law to solutions of aspartic and glutamic acid and to glycine. The data are given in Table XV. The assumption was made that the apparent dissociation constants of these amino acids can be used in place of the true dissociation constants. In the case of the dicarboxylic amino acids this assumption has been shown to be justifiable (32). The data which are given in Table XV show that the dilution law is approximated only at extremely high dilutions. It is of interest to note that whereas the values for K_{Λ} of aspartic and glutamic acids tend to become larger with increasing dilution, the opposite is true for glycine.

10. INFLUENCE OF TEMPERATURE ON IONIC MOBILITY OF ASPARTATE AND GLUTAMATE IONS

The variation of ionic conductance with temperature may be represented by the equation,

$$\Lambda_t = \Lambda_{25} [1 + C(t - 25)] \tag{25}$$

where Λ_t is the ionic conductance or mobility at infinite dilution at t° , and Λ_{25} that at 25°. In the above equation, the quantity C, which is equal to $(d\Lambda/dt)(1/\Lambda_{25})$, is the temperature coefficient. A comparison of the values of the temperature coefficients of aspartate and glutamate ions and certain other ions is given in Table XVI. The values for the amino acid ions lie within the range of the values for the other ions.

TABLE XVI

The Influence of Temperature on Ionic Conductivity or Mobility

Ions	$(\Lambda_0)_{25}^{\circ}$	$(\Lambda_0)_{0}$ °	$1 d\Lambda$
	(120) 20		Λ_{25} dt
Glutamate-	25.10	12.60	0.0198
Aspartate-	27.75	14.20	0.0195
H+	349.99	225.50	0.0146
Na+	50.52	25.80	0.0195
1/2 Ca++	58.82		
1/2 Ba++	64.21	33.00	0.0194

(Miyamoto, S., and Schmidt, C. L. A., Univ. of Calif. Pub. Physiol., 8, 9 (1932).)

Table XVII

The Specific Conductance of Glycine, d,l-Valine, and l-Asparagine

Temperature	Concentration	Specific conductance
	(a) Glycine	
degrees	moles/liter	mhos×106
0	0.077	0.4
	0.196	1.2
	0.328	2.0
	0.512	3.0
	0.640	3.7
	0.800	4.5
	1.000	5.5
25	0.077	1.4
	0.196	3.7
	0.328	6.2
	0.512	9.5
	0.640	11.8
	0.800	14.5
	1.000	17.8
35	0.048	1.4
	0.100	2.9
	0.240	7.0
	0.400	11.5
	0.700	19.5
	1.000	27.1
	(b) d,l-Valine	
0	0.039	0.08
	0.098	0.24
	0.244	0.60
	0.406	1.00
	0.508	1.20
25	0.039	0.4
	0.098	1.1 (a. 1.1)
	0.244	2.7
	0.420	4.5
	0.507	5.4
35	0.036	1.1
	0.061	1.6
	0.151	3.1
	0.252	4.7
	0.419	7.7

713	***	TT 1 /	Continue	~~

Temperature	Concentration	Specific conductance		
	(c) l-Asparagine			
degrees	moles/liter	$mhos\! imes\!10^6$		
25	0.0334	1.68		
	0.0667	2.89		
	0.1000	3.96		
	0.1333	5.10		
	0.1665	6.14		
	0.2000	7.46		

(Mehl, J. W., and Schmidt, C. L. A., J. Gen. Physiol., 18, 467 (1935).)

Due to their low degree of dissociation in the classical sense, the conductivities of the α-monoaminomonocarboxvlic acids are low. It is therefore necessary that only amino acids in a high state of purity be used in conductivity measurements. Even then, too great a reliance cannot be placed on measurements in which the conductivity of a concentrated solution of the amino acid approaches that of the solvent. Even when the values obtained with repeatedly purified samples give reasonable check results. the validity of the results is still not assured. This may indicate that the amount of contaminating substance has reached a constant value. Furthermore, the nature of the proper correction to be applied for the conductivity of the solvent, as shown by Washburn (33), is in doubt. It is possible that the solute influences the dissociation of the impurity (or vice versa) in such a manner that its contribution to the conductivity in the solution is different from that in the solvent alone. Under such circumstances simply subtracting the conductivity of the solvent will not be adequate. The nature of the impurity and its influence will have to be known in order to make a valid correction. The conductivity data which have been obtained by Mehl and Schmidt (34) (see Table XVII) should be accepted with the above reservations in mind.

11. DISSOCIATION CONSTANTS OF AMINO ACIDS FROM CONDUCTIVITY MEASUREMENTS

It has already been indicated in Chapter XI that conductivity measurements do not yield exact values for the dissociation constants of amino acids. Deviations from the laws of perfect solutions,

Table XVIII

Comparison of Specific Conductivity Data* Obtained by Various Workers

Compound	Concentra-	Present work	Walker	Siegfried	Miyamo- to and Schmidt
Asparagine Alanine Glycine	0.0625 M 0.34 M 1.00 M	2.8 6.7 17.8	6.0	23.5 34.7	50.9

* All conductivity values are $\times 10^6$. (Mehl, J. W., and Schmidt, C. L. A., J. Gen. Physiol., 18, 467 (1935).)

which are at present not well defined, and which may possibly be of considerable magnitude even in dilute solutions, are quantitites of uncertain magnitude. Association of the zwitterions may reduce their activity and thus reduce dissociation. It is possible that this effect may be counteracted or even superseded by the effect of the increasing dielectric constant. Viscosity effects will enter, as will possibly also other factors whose relative importance cannot be evaluated accurately. A comparison of the specific conductivity values which have been obtained by various workers for certain amino acids is given in Table XVIII. The divergence between the various data can probably be ascribed to the factors which have just been enumerated.

With due consideration of the uncertainties cited above, Mehl and Schmidt (34) carried out calculations of the molar conductances of certain amino acids (see Table XIX) which show, in a general way, that the calculated values are of the same magnitude

TABLE XIX

Calculated and Determined Molar Conductance* of Glycine and l-Asparagine

Concentration -	Gly	cine	<i>l</i> -Asparagine		
	Calculated	Determined	Calculated	Determined	
moles/liter					
0.05	20.2	18	46.0	46.1	
0.10	18.0	19	35.5	40.0	
0.20	16.5	19.4	30.4	37.0	
0.40	15.6	19.0			
0.60	15.4	18.4			
0.80	15.4	18.1			

^{*} All values given in Table are ×103. (Mehl, J. W., and Schmidt, C. L. A., J. Gen. Physiol., 18, 467 (1935).)

as those which were obtained by direct measurement. Following the procedure of Walker (27), but using a slightly different terminology, the following equations may be set up:

$$\frac{(\mathbf{R}^{-})(\mathbf{H}^{+})}{(\mathbf{R}^{\pm})} = K_{1} = k_{a} \text{ (classical acid dissociation constant)}$$
 (26)

$$\frac{(\mathbf{R}^{+})}{(\mathbf{R}^{\pm})(\mathbf{H}^{+})} = k_2 = \frac{k_b}{k_w} \tag{27}$$

where R⁺=the cation of the ampholyte, R⁻=the anion of the ampholyte, and R[±]=the undissociated ampholyte (chiefly in the zwitterion form). These expressions assume that the activities may be taken as being numerically equal to the concentration. If the concentration of OH⁻ is neglected, since it will be very small in appreciable concentrations of the amino acids under consideration,

$$(H^+) + (R^+) = (R^-)$$
 (28)

In all except very dilute solutions, the concentrations of R⁺ and R⁻ with respect to the total concentration of amino acid may also be neglected, so that

 $(\mathbf{R}^{\pm}) = C \tag{29}$

where C = total concentration of amino acid. Solving for (\mathbf{H}^+) ,

$$(H^{+}) = \sqrt{\frac{CK_1}{1 + CK_2}} \tag{30}$$

which is equivalent to the expression derived by Walker when the concentration of OH⁻ is neglected. When we solve for the concentrations of the amino acid ions and express the specific conductance in terms of these concentrations and the ionic conductances, there is obtained the expression,

$$\overline{L} = \left[\frac{CK_1(\Lambda_H^+ + \Lambda_R) + 2\Lambda_R C^2 K_1 K_2}{\sqrt{CK_1(1 + CK_2)}} \right] \times 10^{-3}$$
(31)

$$\Lambda_{c} = \frac{K_{1}(\Lambda_{H}^{+} + \Lambda_{R}) + 2\Lambda_{R}CK_{1}K_{2}}{\sqrt{CK_{1}(1 + CK_{2})}}$$
(32)

The calculated values for glycine at 25°, which are given in Table XIX, were obtained by use of the following dissociation data: $K_1 = 1.655 \times 10^{-10}$, $K_2 = 224$. The mobility of the glycine zwitterion (see next section), on the basis of McBain and Daw-

son's (35) diffusion data, was taken to be 40.7. This corresponds to a limiting ion conductance of 39.2. For l-asparagine, the limiting ion conductance was taken to be the same as that which was found by Miyamoto and Schmidt (13) for the aspartate ion, viz., 27.75. The dissociation constants used are: $K_1 = 1.38 \times 10^{-9}$, $K_2 = 148$. The agreement between the calculated and determined values in Table XIX is satisfactory only in a qualitative way. The deviations

TABLE XX

The Determined and Calculated Specific Conductance of Aspartic and Glutamic Acid

	Aspar	tic acid	Glutamic acid			
Concentration	Specific co	onductance	Specific conductance			
	Calculated	Determined*	Calculated	Determined*		
moles/liter	$moles/liter$ $mhos imes 10^5$	$hos \times 10^5$ $mhos \times 10^5$		mhos×105		
0.0001	2.46	2.45	1.80	1.78		
0.0002	3.98	3.94	2.77	2.76		
0.0005	7.08	7.05	4.69	4.65		
0.001	10.51	10.67	6.80	6.64		
0.002	14.78	15.61	9.48	9.46		
0.005	23.5	24.3	15.2	14.3		
0.01	29.5	32.7	18.8	18.9		
0.02	38.5	43.4	24.2	25.1		

^{*} Only the data of Miyamoto and Schmidt have been considered in making these calculations, so that, while the values found are comparable with those which have been calculated, any error in the conductivity measurements will introduce a corresponding error in the values calculated for the dissociation constants.

(Mehl, J. W., and Schmidt, C. L. A., J. Gen. Physiol., 18, 467 (1935).)

with increasing concentrations are in the opposite direction from that which might be expected.

Using the values $K_1 = 2.4 \times 10^{-10}$, $K_2 = 209$, and $\Lambda_c = 11.1 \times 10^{-3}$ at 0.075 molar, the mobility of the valine ion is calculated to have a value of 14 which is quite unsatisfactory when compared to the limiting conductances of valerate and caproate ions which are 31 and 29, respectively. On the other hand, the limiting conductance of the glycine zwitterion agrees quite well with the limiting ion conductance of the acetate ion (40.87) when the introduction of the $-NH_2$ group is considered, and the mobility of the glycine ion is taken to be the same as that of the zwitterion.

In the case of the dicarboxylic amino acids (see Table XX) the agreement between the calculated and the determined conduct-

ance values is quite satisfactory in the lower concentrations. In higher concentrations the deviation is not greater than might reasonably be expected. Although, as already indicated, the Ostwald dilution law cannot be strictly applied to solutions of amino acids, measurements can be made in sufficiently dilute solutions so that a simplified theoretical formulation can be applied to the results and the conductivity data used in the calculation of the dissociation constants. It will be assumed that the zwitterion is formed predominantly between the amino group and the carboxyl group closest to it. The expressions for the dissociation of the dicarboxylic amino acids may be written in the following manner:

$$\frac{(R^{-})(H^{+})}{R^{\pm}} = K_{1} \qquad R^{-} = -OOC - R \qquad (33)$$

$$\frac{(R^{+})}{(R^{\pm})(H^{+})} = K_{2} \qquad R^{\pm} = HOOC - R \qquad (34)$$

$$COO^{-}$$

$$NH_{3}^{+}$$

$$COO^{-}$$

$$NH_{4}^{+}$$

$$\frac{(R^{+})}{(R^{\pm})(H^{+})} = K_{2} \qquad \qquad R^{\pm} = \text{HOOC} - R \qquad (34)$$

$$\frac{(R^{-})(H^{+})}{R^{-}} = K_{3} \qquad R^{+} = HOOC - R \qquad (35)$$

and

$$R = -OOC - R$$

$$COO-$$

While these expressions are not complete, they should be adequate for the present purpose. It will also be assumed that the effect of the third dissociation may be neglected, and no attempt will be made to correct for activities or changes in mobility in more concentrated solutions. Under these conditions the following expressions are obtained:

$$K_2(H^+)^3 + (CK_2 + 1)(H^+)^2 + K_1(H^+) - CK_1 = 0$$
 (36)

$$(R^{\pm}) = \frac{C}{1 + K_1/(H^+) + K_2(H^+)}$$
 (37)

where C = total concentration of the amino acid.

Since the conductance of these solutions is largely due to the hydrogen ions in the lower concentrations, a first approximation of the hydrogen ion concentration can be made by dividing the specific conductance by the sum of the mobilities of the hydrogen and amino acid ions (since there will be at least one amino acid ion for each hydrogen ion), and multiplying by 10^3 . If this value is then substituted in equation (36) for the two lowest concentrations, the equations may be solved simultaneously for K_1 and K_2 . Having a rough approximation of the value of the constants, the corresponding approximations for \mathbb{R}^{\pm} and \mathbb{R}^{+} may be made. At any

TABLE XXI

The Acid Dissociation Constants of Certain Substances

Substance	K_{A_1}	K_{A_2}	Reference
Glycine	4.47 ×10 ⁻³		(1)
Acetic acid	1.753×10 ⁻⁵		(2)
Aspartic acid	6.45×10^{-3}	1.24×10^{-4}	Author's work
Succinic acid	6.3×10^{-5}	1.5 ×10 ⁻⁶	(3)
Asparagine	6.75×10^{-3}	<u> </u>	(4)
Glutamic acid	8.7 ×10 ⁻³	4.45×10^{-5}	Author's work
Glutaric acid	4.8 ×10 ⁻⁵	3.2 ×10 ⁻⁶	(3)

(1) Owen, B. B., J. Amer. Chem. Soc., 56, 24 (1934).

(2) MacInnes, D. A., Shedlovsky, T., and Longsworth, L. G., Chem. Rev., 13, 29 (1933).

(3) Simms, H. S., J. Amer. Chem. Soc., 48, 1251 (1926).

(4) Kirk, P. L., and Schmidt, C. L. A., Univ. of Calif. Pub. Physiol., 7, 57 (1929).

(Mehl, J. W. and Schmidt, C. L. A., J. Gen. Physiol., 18, 467 (1935).)

concentration, that part of the conductivity which is not due to the hydrogen ion and an equal concentration of R^- , will be due to the concentration of R^+ present and an equal concentration of R^- . If then, having the approximate value for the concentration of R^+ , it is multiplied by the limiting conductance value of this ion, the proper factor, and the resultant product is doubled, a correction to be subtracted from the experimental conductance is obtained in order to make a second approximation of (H^+) . By such a series of approximations it is finally possible to arrive at values for K_1 and K_2 which give a satisfactory agreement with the conductivity data at lower concentrations. In this manner the values $K_1 = 4.45 \times 10^{-5}$ and $K_2 = 115$ were obtained for glutamic acid; and $K_1 = 1.24 \times 10^{-4}$ and $K_2 = 155$ for aspartic acid at 25°. The value for the second acid dissociation constant for aspartic acid was

calculated to be 6.45×10^{-3} , and for glutamic acid 8.7×10^{-3} (see Table XXI). The accuracy of the values for K_1 should be quite good. The values probably do not deviate more than 5 per cent from the true constants. The accuracy with which K_2 may be calculated is considerably less since its effect is not so great as that of K_1 , particularly in the lower concentrations where the results should be most dependable. The values for K_2 may be 20 per cent in error. The above acid dissociation constants are not in good

Table XXII

Specific Conductivity of Certain Proteins in Glacial Lactic and Acetic Acids

		Edestin i aci					n in acetic acid	
Concen- tration	Specific conduc- ti vi ty	Concen- tration	Specific conduc- tivity	Concen- tration	Specific conductivity	Concen- tration	Specific conductivity	
gm. per liter of solvent	mhos ×10⁵	gm.perliter of solvent	mhos ×10⁵	gm.perliter of solvent	$^{mhos}_{ imes 10^5}$	gm. per liter of solvent	$mhos imes 10^5$	
0	5.14	0	5.25	0	7.37	0	0.01 (about)	
2.80	5.06	2.85	5.24	4.30	7.48	27.0	1.22	
5.65	4.95	5.70	5.23	8.60	7.53	36.0	1.97	
8.45	4.93	8.55	5.30	13.00	7.75	45.0	2.75	
11.25	4.90	11.40	5.34	17.30	8.03	54.0	3.64	
14.05	4.88	14.25	5.46	21.60	8.11	63.0	4.71	
16.90	4.87	17.10	5.49	26.00	8.52	72.0	5.79	
19.70	4.86	19.95	5.53	30.30	8.82			
22.50	4.96	22.80	5.56	36.10	9.03			
25.30	4.97			Mara (A. D.)				
28.15	5.04							

(Greenberg, D. M., and Larson, C. E., J. Phys. Chem., 39, 665 (1935).)

agreement with the values obtained by electrometric titrations, though they are not less divergent than values which have been obtained by different workers.

The equivalent conductances of several concentrations of KCl in 0.05 and 0.1 molal alanine solutions at 25° have been determined for the purpose of ascertaining the contribution of the zwitterion to the ionic atmosphere. These values were plotted against the square root of the KCl concentration in order to extrapolate to an infinite dilution of KCl. This was likewise done for urea. The ratios of these values for limiting conductances to that in water, Λ_0/Λ_0 (H-0), for 0.05 and 0.10 molal alanine and 0.05 molal urea

are, respectively, 0.995, 0.984, and 1.00. The relative fluidities, $\nu/_{\rm H,0}$, at 18° are, respectively, 0.99, 0.98, and 1.00. It is evident that a change in viscosity of the solution is in itself sufficient to account for the greater part of the effect of alanine, and that there is no

TABLE XXIII

Conductivity of Casein in Formic Acid

Concen- tration	X Specific conduc- tivity*	Equivalent concentration $N = 990$ (mean of dye titration and hexone base content)	Δ	Concen- tration	X Specific conduc- tivity†		Λ
S. Hanne		Casein I			C	asein II	
gm. per liter of solvent	mhos ×10 ⁵		mhos	gm. per liter of solvent	$mhos \ imes 10^{5}$		mhos
4.55	17.3	0.0046	37.7	1.90	7.2	0.0019	38.0
9.30	37.6	0.0094	40.0	4.70	17.3	0.0047	36.8
13.90	55.1	0.0140	39.3	9.45	39.9	0.0095	42.0
18.55	73.2	0.0187	39.1	14.15	56.6	0.0143	39.6
23.20	95.8	0.0235	41.7	18.90	74.6	0.0191	39.1
25.10	102.8	0.0254	41.5	23.60	91.6	0.0239	38.3
27.85	110.6	0.0282	39.2	28.30	109.1	0.0286	38.2
32.50	121.1	0.0328	36.7	33.10	126.7	0.0334	38.0
39.10	138.8	0.0395	35.2	37.85	140.4	0.0382	36.8
46.40	169.8	0.0470	36.2	47.20	170.5	0.0477	35.8
55.70	200.0	0.0563	35.5	56.65	198.4	0.0572	34.7

^{*} Specific conductivity of solvent = 14.0×10^{-5} mhos has been subtracted from the total.

(Greenberg, D. M., and Larson, C. E., J. Phys. Chem., 39, 665 (1935).)

effect which is at all comparable to that of an electrolyte. The results are in agreement with those of Failey (36).

Greenberg and Larson (16) have reported conductivity studies on gelatin, easein, and edestin which were dissolved in glacial acetic, lactic, and formic acid. Several of their data are given in Tables XXII to XXV. The values for the equivalent conductivities of the three proteins are nearly the same. The results lead to the conclusion that the proteins form ionizable salts in formic acid which are analogous in electrochemical behavior to the alkali and alkaline earth formates in formic acid. On the other hand, the conductivity of glacial acetic and of lactic acid is increased but little on addition

[†] Specific conductivity of solvent = 10.15×10^{-5} mhos has been subtracted from the total.

of these proteins, respectively. Evidently, these acids do not react with proteins to form protein ions.

TABLE XXIV

Conductivity of Edestin in Formic Acid

Concen- tration	X Specific conductivity*	Equivalent concentration $N=640$ (dye titration value)	À	Equivalent concentration $N = 750$ (hexone base content)	A
gm. per liter of solvent	mhos×10 ⁵		mhos		mhos
4.75	29.7	0.0074	40.1	0.0063	47.2
9.50	55.9	0.0148	37.8	0.0127	44.0
14.25	85.4	0.0223	38.3	0.0190	45.0
19.00	115.3	0.0297	38.9	0.0254	45.4
23.80	144.7	0.0372	38.9	0.0318	45.5
28.50	165.1	0.0445	37.1	0.0380	43.5
33.30	195.7	0.0518	37.8	0.0444	44.0
38.10	216.8	0.0595	36.5	0.0508	42.7

^{*} Specific conductivity of solvent = 10.15×10^{-5} mhos has been subtracted from total.

(Greenberg, D. M. and Larson, C. E., J. Phys. Chem., 39, 665 (1935).)

Table XXV

Conductivity of Gelatin in Formic Acid

Concentration	X Specific conductivity*	Equivalent concentration $N = 950$ (Mean of dye titration and hexone base content)	Ā
gm. per liter of solvent	mhos×10 ⁵		mhos
9.0	37.5	0.0095	39.5
18.0	77.0	0.0190	40.5
27.0	116.1	0.0285	40.8
36.0	151.9	0.0379	.40.0
45.0	185.4	0.0475	39.0
54.0	217.1	0.0568	38.2

^{*} Specific conductivity of solvent = 10.8×10^{-5} mhos has been subtracted from the total.

(Greenberg, D. M., and Larson, C. E., J. Phys. Chem., 39, 665 (1935).)

12. MOBILITIES OF AMINO ACID AND PROTEIN IONS

Reference has already been made to the calculation of ionic mobility from transference data. The ionic mobility may, in the

case of solutions of amino acid and protein salts, be determined by extrapolating the equivalent conductance to infinite dilution. The relationship between the transport number and the ionic mobilities in a solution is given by the equation,

$$T_{\rm anion} = \frac{\Lambda_{\rm 0~anion}}{\Lambda_{\rm 0~anion} + \Lambda_{\rm 0~cation}},$$
 (38)

in which Λ_0 is the mobility or equivalent conductance at infinite dilution. Comparison between the values which were obtained

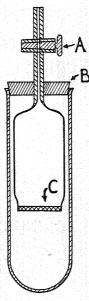


Fig. 18. Sintered glass diffusion cell.

A.—Stock-cock.

B.—Rubber stopper.

C.—Sintered glass disk.

(Mehl, J. W., Thesis, Univ. of Calif., 1936.)

from transport experiments and from conductivity data is shown in Table XII. In general, the values which are obtained by extrapolation of conductivity data and those which are calculated from transport numbers are in good agreement.

In the case of monoaminomonocarboxylic acids, due to the fact that, in the classical sense, they are very weak electrolytes, it is not feasible to carry out transport experiments. Extrapolation of conductivity data to infinite dilution involves large errors. The mobility of the amino acid, or the zwitterion, may be determined

by diffusion measurements. This consists in estimating the rate of diffusion of the amino acid from a solution of known concentration into distilled water at a given temperature. The two phases are separated by a sintered glass disc. Fig. 18 illustrates the apparatus in a simple form.

In the following discussion the term " Λ_0 " is used to express the mobility of the zwitterion. Actually its significance is not the same

Table XXVI

Mobilities of Amino Acids from Diffusion Measurements

Amino Acid	30°	25°	10°	1.0°
d, l-Alanine	34.8	31.3	22.0	16.6
l-Asparagine	32.4	29.5	20.6	
l-Aspartic Acid		27.81		
d-Glutamic Acid		25.1^{1}		
Glycine	40.7	36.8	25.4	19.6
d, l-Proline	34.6	31.0	21.4	16.1
d, l-Valine	30.3	27.1	18.5	14.0

Temperature coefficients.

d, l-Alanine. (1° to 10°) 0.042

(1° to 30°) 0.046

Glycine: (1° to 10°) 0.040 (1° to 30°) 0.045

d, l-Proline: (1° to 10°) 0.041

d, l-Valine: (1° to 30°) 0.048 (1° to 10°) 0.041 (1° to 30°) 0.0485

¹ Mobility of the ion from conductivity data (Miyamoto, S., and Schmidt, C. L. A., *Univ. of Calif. Pub. Physiol.*, 8, 9 (1932).)

(The above calculations are based on data obtained by (Mehl, J. W., and Schmidt, C. L. A., *Univ. of Calif. Pub. Physiol.*, 8, 165 (1937); Mehl, J. W., Thesis, Univ. of Calif., (1937).)

as when this term is used to designate the mobility of a dissociated ion. " Λ_0 " represents the mobility of an hypothetical univalent ion having a diffusion coefficient which is equivalent to that of the amino acid when it is acted upon by an electrical force of one volt per cm. We shall designate the determined diffusion coefficient, in cm.² per day, by D. D_0 expresses the same quantity at infinite dilution. Since a field of one volt acting over an area of one cm. = 10^8 electromagnetic units, and the charge on an ion = 1.592×10^{-20} E.M.U., the force acting on a single ion in a field of one volt per cm. = 1.592×10^{-12} E.M.U. The force acting on one gm.

mole = $1.592 \times 10^{-12} \times$ the number of ions in a gm. mole, (6.06 $\times 10^{23}$), or, 9.65×10^{11} dynes. The velocity under a force of one dyne = $U/9.65 \times 10^{11} = D/RT$, where U, the electrical mobility in cm.²/volt/sec., is equal to $\Lambda_0/96500$. From this it follows that

$$D = \frac{RT\Lambda_0}{96500 \times 9.65 \times 10^{11}}$$

or,

$$\Lambda_0 = \frac{D \times 9.31 \times 10^{16}}{RT} \tag{39}$$

The data given in Table XXVI indicate that the mobilities, at 25°, of the five given compounds are appreciably greater than those of aspartic and glutamic acid. The mobilities of the latter amino acids were determined by conductivity measurements (37). The temperature coefficients are also considerably larger. The temperature coefficient of glycine does not follow a strictly linear function.

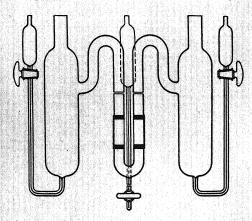


Fig. 19. Apparatus for the determination of electrical mobility. (Tiselius, A., Nova Acta Reg. Soc. Sci. Upsaliensis, 7, ser. IV, No. 4, 1930).

The mobilities of protein ions may be determined by electrophoresis. The values so obtained are actual velocities. They are expressed in cm.²/volt/sec., and not in terms of electrical mobility. Extensive measurements by this method have been carried out by Svedberg and Tiselius (38). For this purpose a three-compartment cell, shown in Fig. 19, is used. The center compartment is a U-tube fitted with quartz windows through which ultraviolet light can be passed so that the movement of the protein solution can

be followed photographically. Silver-silver chloride electrodes in saturated potassium chloride solution are employed. The proteins are dissolved in buffer solutions of various pH values. The protein solution is placed in the center compartment. The calculations are made as follows. If the current is i ampere, the conductivity of the solution at a certain place in the U-tube is χ , the cross section here being q cm.², then the potential gradient F volt/cm. at this place is given by the equation,

$$F = \frac{i}{q\chi} \tag{40}$$

If the mobility is u, the distance of migration in the time t seconds is

$$F \times u \times t = u \times \frac{it}{q_X} \tag{41}$$

The best way is to measure the quantity of electricity, *it*, passed. Since reversible electrodes are used, *i* is rather constant. Hence it may be read directly at various times and the mean value used. Certain of the data obtained by Tiselius are given in Table XXVII. The essential thing to note in these experiments is that the mobility of the protein varies with the pH of the solution.

These experiments have, more recently, been repeated and extended by Abramson (39). He measured the migration of microscopically visible quartz particles, which were covered with a film of protein, in buffer solutions of varying pH, using a modified Northrop-Kunitz (40) micro-electrophoresis cell. From theoretical considerations it was shown that

$$Q = 6\pi \eta vr \left[(r\sqrt{\mu} \times 0.33 \times 10^8) + 1 \right]$$
 (42)

where Q = the charge in the particle, C = a number which depends upon the size and shape of the small particle in a particular electrode solution, η = coefficient of viscosity, r = radius of the particle, v, its velocity, and μ = ionic strength. The reader is referred to the original for the development of this equation. When the activity of the hydrogen ion is varied and the ionic strength is kept sufficiently constant $(C = \sqrt{\mu})$, then,

$$Q = 6\pi \eta vr \left[(Cr \times 0.33 \times 10^8) + 1 \right] \tag{43}$$

This equation states the conditions for which any given molecule of radius r moving with a velocity v in a field of unit potential

Table XXVII

Mobilities of Proteins at 20° from Electrophoresis Experiments

Experi- ment Number	Protein	pН	Direction of Migration	${ m Mobility} \ { m cm^2/sec./volt} \ { m imes 10^5} \ { m (average)}$
1	Egg albumin	4.27	C	3.1
	(0.35 per cent)	4.44	C	1.4
		4.68	A	1.4
		4.90	A	3.4
		5.04	A	4.9
		5.27	A	6.6
2	Serum albumin	4.21	C	6.0
	(0.23 per cent)	4.40	C	4.4
		4.68	C	2.0
		4.95	A	0.7
		5.13	A	2.4
		5.34	A	4.2
3	Helix Hemocyanin	4.36	C	5.8
	(0.15 per cent)	4.67	C	2.9
		5.39	A	2.3
4	Bence Jones protein	4.37	C	4.7
	(0.15 per cent)	4.67	C	2.9
		5.34	A	0.9
		5.65	A	2.6
5	C-Phycocyan	4.07	C	7.6
	(0.18 to 0.20 per cent)	4.34	C	4.4
	•	4.68	C	0.7
		4.87	' A	0.9
		4.97	A	1.6
		5.35	A	3.7
6	Egg albumin	4.27	C	3.6
		4.51	C	1.1
		5.11	A	3.0
7	Egg albumin	5.98	A	11.0
		6.93	A	14.6
		7.71	A	18.0
8	C-Phycocyan	4,26	C	5.6
		5.70	A A	5.9

Note: Experiments Nos. 1 to 5 were carried out with acetic acid-sodium acetate buffer, No. 6 with a barium acetate-acetic acid buffer, No. 7 with a phosphate buffer, and No. 8 was carried out in an unbuffered solution. It will be noted that the mobility of the protein ion varies with the pH of the solution and depends somewhat on the valency of the cation.

C = cathodic

A = anodic

(Tiselius, A., Nova Act. Reg. Soc. Sci. Upsaliensis, 7, Ser. IV, No. 4, (1930).)

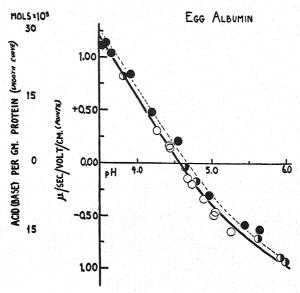


Fig. 20. The open circles are values of electric mobility of dissolved egg albumin obtained by Tiselius. The closed and half-closed circles are similar data for egg albumin studied under similar conditions but adsorbed on microscopically visible quartz particles. The data of Loeb have been used to plot the titration curve. It is evident that the mobility and titration curves belong to the same family, so that over this range of pH, mobility is proportional to the acid (base) bound. The dotted line indicates the very slight shift in electrophoretic mobility between adsorbed and dissolved protein.

(Abramson, H. A., J. Gen. Physiol., 15, 575 (1932).)

gradient, has the charge, Q, proportional to the electrophoretic velocity. Collecting constants,

$$Q = v(C' + C'') \tag{44}$$

where

$$C' = (6\pi\eta r^2\sqrt{\mu} \times 0.33 \times 10^8)$$

and

$$C^{\prime\prime} = (6\pi\eta r)$$

On the basis of the assumptions that (a) the change in viscosity and the effective radius, r, with change in hydrogen ion activity is negligible; (b) the protein salt is completely dissociated; (c) the reaction of the protein with ions other than hydrogen or hydroxyl is negligible; (d) only uni-univalent strong electrolytes are considered; (e) the values of the dielectric constant and viscosity of the medium can be used in place of the unknown values in the

double layer; (f) in plotting mobility of the same protein against pH, the salt present influences the protein in the same way at all investigated values of pH, the following rule can be given on the basis of equation (44): in solutions of the same ionic strength, the electric mobility of the same protein at different hydrogen ion activities should be directly proportional to the number of hydrogen or hydroxyl ions which are bound by each molecule.

The change in mobility of egg albumin with pH is graphically represented in Fig. 20, and the interpolated data for egg albumin and serum albumin are given in Table XXVIII.

Table XXVIII

The Electric Mobilities of Two Proteins in M/50 Acetate Buffer

pН	v Egg albumin	v Serum albumin
	μ/sec./volt/cm.	μ/sec./volt/cm.
3.50	1.20	
3.70	0.96	1.30
3.90	0.74	1.05
4.10	0.53	0.79
4.30	0.30	0.55
4.50	+0.077	0.34
4.57	0.00	
4.70	-0.014	+0.16
4.88		0.00
4.90	0.33	-0.044
5.10	0.47	0.27
5.30	0.61	0.39
5.50	0.72	0.50
5.70	0.83	0.63
5.90	0.92	

(Abramson, H. A., J. Gen. Physiol., 15, 575 (1932); See also Moyer, L. S., and Abramson, H. A., J. Biol. Chem., 123, 391 (1938).)

From these data, as well as from data obtained on other proteins, it has been shown that the mobility and titration curves of proteins are congruent within the limits of experimental error. This follows from equation (44). In plotting the titration curve of the protein, it is essential that the amount of acid or base bound per unit weight of protein be plotted.

13. E-POTENTIAL

When a potential difference is applied between two liquids which are separated by a porous membrane, a flow of liquid occurs in the direction of the negative electrode. This phenomenon is known as electro-endosmosis. Cataphoresis denotes the movement of small particles suspended in a liquid when a potential difference is set up between two electrodes dipping in the liquid. According to the Helmholtz-von Smoluchowski (41) theory of cataphoresis, the equation for V_p , the cataphoretic velocity of a particle relative to a given medium is given by

$$V_p = \frac{1}{4\pi} \frac{X \epsilon \zeta}{\eta} \frac{CX}{\eta} \tag{45}$$

where X = field strength, ϵ = dielectric constant of the medium, ζ = electrokinetic potential, η = viscosity of the medium, and C = a constant. All of the terms are expressed in c.g.s. electrostatic units. The ζ -potential designates the potential gradient in the double layer of two phase boundaries in tangential motion to each other. The double layer may be likened to a condenser since a negatively charged layer is separated from a positively charged layer at a distance, δ , from each other. For such a condenser, $\zeta = 4\pi \delta e/D$, where e = quantity of electricity per square centimeter of wall.

It has been shown by Abramson (42) and by Daniel (43) that the ζ -potential of inert microscopic particles covered by protein films is the same as that calculated for the ζ -potential of the wall of the electrophoresis cell when coated with the same protein. In other words, the ratio, R, of the electro-osmotic mobility, u, to the electrophoretic mobility, v, is unity. The size and shape of such particles do not influence their mobilities, which depend entirely on the protein film. The ζ -potential may also be determined from streaming potential measurements. These potentials arise from the flow of liquids through capillaries. The ζ -potential may be calculated by means of the equation,

$$\zeta = \frac{4\pi\eta K v}{i\epsilon} \tag{46}$$

where K =specific conductivity, i = current, and v =flow of liquid. Bull (44) has shown that the value for R obtained from protein-covered surfaces is unity when ζ was determined by electrophoresis, electro-osmosis, and streaming potential. Moyer and Abramson (45) have more recently found that the value for R of protein-coated surfaces is very close to unity, even in very dilute solutions of electrolytes.

14. METALLIC ELECTRODES

When two phases are brought into contact a difference in electrical potential is set up, the magnitude of which is a function of the nature of the components, their physical states, their concentrations, the temperature, and the pressure. In accordance with this statement differences of potential will result when (a) two different metals are placed in a solution of an electrolyte, (b) two different metals are placed in solutions of two different electrolytes, (c) the same metal is placed in solutions of two different electrolytes, and (d) the same metal is placed in two solutions of the same electrolyte, but differing in concentration. It is understood that all of these cells are appropriately connected so that the electrical circuit is completed.

For purposes of studying the electrochemical behavior of amino acids and proteins, concentration cells conforming to the type which is given under (d) are of great interest. The electromotive force which is developed in a concentration cell is a function of the ratio of the concentrations of the electrolyte in the two parts of the cell. Knowing the activity of an ion in one part of the cell and the electromotive force, the activity of the corresponding ion in the other half of the cell can readily be calculated. Since the properties of a cell of type (d) can often be reconstructed from a cell of type (b), it is common practice to construct a cell of two different electrodes, one of which serves as a reference electrode. Thus a calomel cell is usually employed as a reference electrode in hydrogen electrode measurements in place of a half cell in which hydrogen gas is in equilibrium with a solution of known hydrogen ion activity.

There are comparatively few metallic electrodes which lend themselves to the estimation of the activity of the metallic ion in either solutions of the inorganic salts or solutions of amino acids or proteins. Silver is one of those which can be so used. From a technical standpoint the silver electrode should be coated with either a deposit of sponge silver or silver chloride. Silver ion activity measurements in protein solutions have been carried out by Pauli and Matula (46) and, more recently, by Goigner and Pauli (47).

The technique consists in measuring the silver ion activity in silver nitrate solutions with and without the addition of protein. The silver electrode consists of a 1 mm. silver wire freshly coated with electrically deposited silver which is then converted to the chloride by electrolysis in hydrochloric acid or ammonium chloride.

For this purpose the procedure of Katsu (48) is recommended. The other half of the cell may be either a silver electrode immersed in 0.1 N silver nitrate solution or a normal calomel electrode with a bridge consisting of a saturated potassium nitrate solution. The silver ion activity is determined on the basis of electromotive force measurements with the aid of the Nernst formula. At 20°

$$p_{\rm Ag}^{+} = \log \frac{1}{a_{\rm Ag}} \frac{\pi - \pi_0}{0.0581} \tag{47}$$

where π is the E.M.F. determined in the protein-containing silver

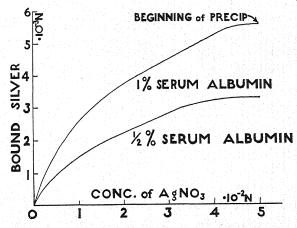


Fig. 21. Relation between the amount of silver nitrate added and the amount of silver bound by certain proteins.

(Goigner, E., and Pauli, W., Biochem. Z., 235, 271 (1931).)

solution and π_0 that in a solution of known silver nitrate concentration. The activity coefficient of the silver ion in a 10^{-2} N AgNO₃ solution at 20° is 0.902 and the E.M.F. of a silver electrode in this solution is 0.408 volt. Hence

$$\pi_0 = -0.408 - 0.0581 \log \frac{1}{0.902 \times 10^{-2}} = -0.5268$$

$$\log \frac{1}{a_{Ag}} = p_{Ag} = \frac{0.5268 - \pi}{0.0581}$$
(48)

The data which are given in Table XXIX are illustrative of the results which were obtained. Fig. 21 shows the relationship between the silver nitrate concentration and the amount of silver which was

or

Table XXIX

Silver Ion Activities in Silver Nitrate Solutions With and Without the Addition of Serum Albumin (1 per cent)

Normality AgNO ₃	Activity coeff. of Ag+	Activity of Ag+ in AgNO ₃ sol.	Activity of Ag+ in presence of protein	Conc. of Ag bound by protein	
×10 ²		×10³	×10³	×10³	
0.5	0.932	4.66	3.10	1.67	
1.0	0.902	9.02	6.76	2.51	
2.0	0.854	17.08	13.96	3.65	
3.0	0.818	24.54	20.84	4.52	
4.0	0.791	31.64	27.42	5.33	
5.0	0.767	38.33	34.04	5.59	

(Goigner, E., and Pauli, W., Biochem. Z., 235, 271 (1931).)

bound in two different concentrations of serum albumin. It is evident that the amount of silver combined with the protein is not proportional to the concentration of serum albumin.

These workers also carried out conductivity studies on solutions of silver nitrate with and without the addition of serum albumin. A correction for the interfering action of the protein due to the free mobility of the silver ions was applied to the conductivity measurements. For this purpose conductivity measurements were carried out on a potassium chloride solution with and without the addition of serum albumin. The data, which are given in Table XXX, when plotted, show that the amount of silver which is bound to the pro-

Table XXX

Conductivity of Silver Nitrate Solutions With and Without the Addition of Serum Albumin (1 per cent)

Normality AgNO3	(a) Conductivity AgNO ₃	(b) Conductivity AgNO ₃ +Serum Albumin	(a) — (b)	(c) Correction	(a) - (b) - (c)
×10²	Reciprocal ohms×10³	Reciprocal ohms×103	×10 ⁴	Reciprocal ohms×105	×104
0.5	0.635	0.493	1.42	1.2	1.30
1.0	1.247	1.026	2.21	2.5	1.96
2.0	2.424	2.091	3.33	4.8	2.85
3.0	3.549	3.107 -	4.42	7.1	3.71
4.0	4.672	4.140	5.32	9.3	4.39
5.0	5.745	5.137	6.08	11.5	4.93

(Goigner, E., and Pauli, W., Biochem, Z., 235, 271 (1931).)

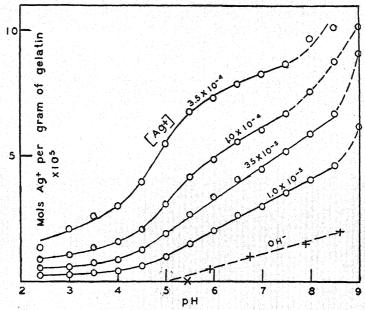


Fig. 22. Change in combination of silver ion and gelatin with pH. Crosses represent combination of hydroxyl ion and gelatin, on one-tenth the scale.

(Carroll, B. H., and Hubbard, D., U. S. Bureau of Standards, J. Res., 7, 811 (1931).)

tein increases with increasing concentration of silver nitrate and tends to reach a maximum value. In this respect the results are analogous to those which were obtained by the use of the silver electrode.

Additional experiments were carried out by adding silver oxide to a one per cent serum albumin solution. After shaking one hour

Table XXXI
Silver Bound on Addition of Silver Oxide to Serum Albumin (1 per cent)

	(a)	(b)	Active Ag.		Inactive Ag.	
	Conduc- tivity	Conc. of Ag.	(e) <i>p</i> _{Ag} +	(d) a _{Ag} +	(e) (b) -(d)	Per cent (b)/(e)
Harris Sala	Reciprocal ohms×104	N×10³		×10 ⁴	×10³	11000
Mixture I	1.34	16.95	3.014	9.68	15.98	94.3
II	1.15	11.42	3.117	7.64	10.66	93.3
III	0.76	8.19	3.306	4.94	7.70	94.0

the excess of silver oxide was filtered off. Estimations of the conductivity, silver ion activity, and concentration of silver were carried out on the filtrate. The data are given in Table XXXI.

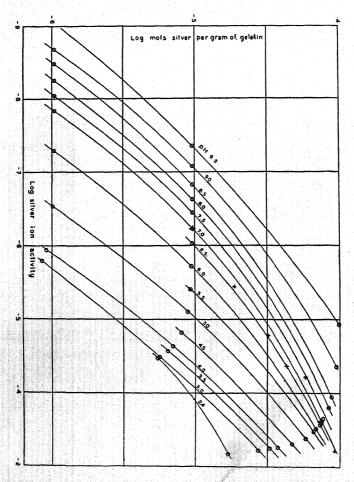


Fig. 23. Combination of silver ion and gelatin at 30°. Circles represent determinations at a concentration of 1.00 gm. per 100 ml.; crosses, determinations at other gelatin concentrations and pH 7.

(Carroll, B. H., and Hubbard, D., U. S. Bureau of Standards, J. Res., 7, 811 (1931).)

It is evident that about 94 per cent of the silver is bound to the protein in a non-ionic form. Donnan equilibrium measurements which were carried out on mixture III showed that the activity coefficient of the silver protein complex was 0.022. It is of interest to note that when the concentration of silver hydroxide is 1.7

 $\times 10^{-2}$ N the amount of silver which is bound by one gram of serum albumin is 159.8×10^{-5} gm. equivalents, while the maximum alkali binding power of this protein is 160×10^{-5} gm. equivalents.

The experiments of Goigner and Pauli (47) indicate that the amount of silver which is bound per unit amount of serum albumin is considerably greater when silver oxide is added than on the

TABLE XXXII

Activity of Silver Ion in Mixtures of Silver Nitrate and Gelatin

1.00 gm. gelatin per 100 ml. in all mixtures; silver nitrate and hydrogen ion concentrations varied

Concentra- of AgNO:			Activity	of silver ion	in mixture,	at pH		
in mixture, N	2.4	3.0	3.5	4.0	4.5	5.0	5.5	6.0
$ \begin{array}{c} 1.00 \times 10^{-8} \\ 9.70 \times 10^{-5} \\ 1.04 \times 10^{-5} \end{array} $	3.42×10 ⁻⁵ 3.3		5.60 ×10 ⁻⁴ 2.70 ×10 ⁻⁵		4.90×10 ⁻⁴ 1.53×10 ⁻⁵	4.1 ×10 ⁻⁴ 7.9 ×10 ⁻⁸ 2.9 ×10 ⁻⁷	3.3×10 ⁻⁴ 3.9×10 ⁻⁶	3.0×10 ⁻⁴ 1.9×10 ⁻⁶ 5.1×10 ⁻⁸
	ration of		Ac	tivity of silv	er ion in mi	xture, at p	н	
AgNO; in mixture, N		6.5	7.0	7.5	8.0	8.5	9.0	9.5
$ \begin{array}{c} 1.00 \times 10^{-3} \\ 9.70 \times 10^{-5} \\ 1.04 \times 10^{-5} \end{array} $		2.65×10 ⁻⁴ 9.40×10 ⁻⁷	2.42×10 ⁻⁴ 6.00×10 ⁻⁷ 1.45×10 ⁻⁸	3.60×10 ⁻⁷	1.55×10 ⁻⁴ 2.40×10 ⁻⁷ 3.30×10 ⁻⁹	1.1×10 ⁻⁴ 1.5×10 ⁻⁷ 3.3×10 ⁻⁹	4.3 ×10 ⁻⁵ 8.4 ×10 ⁻⁸ 2.1 ×10 ⁻⁹	1.17 ×10 ⁻¹ 4.00 ×10 ⁻¹ 9.00 ×10 ⁻¹

Silver Ion Activity in Mixtures of Silver Nitrate and Gelatin pH constant at 7.0; gelatin and silver nitrate concentrations varied

Concen-	Silver ion activity				
tration of gelatin, gm. per 100 ml.	Silver nitrate 9.7×10 ⁻⁴ N	Silver nitrate 9.7×10 ⁻⁵ N			
1.00	2.4×10 ⁻⁴	6.0 ×10 ⁻⁷			
0.50	5.1×10-4	3.6 ×10-6			
0.25	7.0×10~4	1.65×10-5			
0.125	7.8×10 ⁻⁴	4.3 ×10 ⁻⁵			
0.063	8.7×10~4	6.1 ×10 ⁻⁵			

(Carroll, B. H., and Hubbard, D., U. S. Bureau of Standards, J. Res., 7, 811 (1931).)

addition of the same amount of silver nitrate. Analogous results were obtained in the case of glycine. This, as will be shown presently, may be due to differences in pH.

Carroll and Hubbard (49) have studied the influence of pH on the silver combining capacity of gelatin by means of the silver electrode. Their data are given in Figs. 22 and 23 and Table XXXII. The data show that the amount of silver ion combined with unit weight of gelatin increases with increasing pH. The amount of silver which is combined with gelatin at the isoelectric point is a minimum, but the value does not fall to zero. At cor-

responding activities, gelatin combines with many times as much hydroxyl as silver ion. No appreciable combination between $Ag(NH_3)^+_2$ and gelatin was found. The authors suggest that silver ion combines with the free amino groups of the protein molecule to form silver ion-amino complexes. In line with the zwitterion concept the number of $-NH_2$ groups should increase with increasing pH and hence increasing amounts of silver should be bound until a maximum is reached.

A copper electrode was employed by Ettisch, Sachsse and Beck (50) for the purpose of studying the activity of the copper ion in solutions of serum albumin and serum globulin which were dissolved with the aid of sodium hydroxide. Due to the high concentrations of alkali which were employed, it is difficult to interpret their data quantitatively. However, differences in the copper-binding capacity of the two proteins were found.

Further discussion of heavy metal-protein complexes is given in the next chapter.

15. AMALGAM ELECTRODES

Amalgam electrodes have been employed for the estimation of the activities of certain of the alkali and alkaline earth ions in protein and amino acid solutions. A satisfactory calcium electrode has, however, not yet been devised. As an example of the application of amalgam electrodes the work of Kirk and Schmidt (51) may be cited. They used sodium and barium amalgam electrodes. The amalgam is prepared by electrolysis from washed and distilled mercury and a saturated solution of sodium or barium hydroxide. The design of the electrode vessel is shown in Fig. 24.

When solutions of low concentration, comparable to that of biologically important fluids, are used, two-phase saturated amalgams cannot be employed since the rate of reaction of the concentrated amalgams is so great that reliable measurements cannot be made. Dilute amalgams are therefore used. Reproducible electromotive force measurements can be made in quite dilute solutions. The amalgam half-cell is measured against a 1.0 M potassium chloride calomel cell, and connected with it by a saturated KCl bridge.

The solution to be measured is placed in a vessel with a side arm from the bottom, which continuously removes the spent amalgam, thus preventing it from reacting and concentrating the solution. In solutions of moderate concentrations the speed of dropping has no effect, but in concentrations of 0.01 N and below, it is necessary that the amalgam be permitted to flow rather rapidly in order to obtain steady and reproducible readings. Protein-containing solutions are more difficult to measure, due to the more rapid decomposition of amalgam.

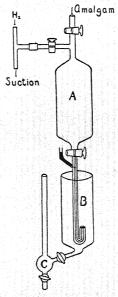


Fig. 24. Shows the design of the sodium and barium amalgam electrode. A represents the reservoir in which the amalgam is stored under dry hydrogen. B is the vessel in which the solution being studied is kept. C is the reservoir in which the amalgam which has been used is kept to prevent reaction with the solution in B. The stop-cock between B and C is kept slightly open during the flow of amalgam, in order to drain it into C.

(Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

The cells whose potentials are to be measured are then of the type,

Me Hg_x (dilute) | solution (containing Me ions at concentration c)

||saturated KCl|| 1.0 N KCl | 1.0 N KCl | HgCl | Hg

where Me is either Na or Ba. If it is assumed that the saturated KCl bridge keeps the diffusion potential at some constant value when solutions of varying concentrations are used, the difference of potential of two cells varying only in the value of c should give the potential of a concentration cell without transference of the type,

Me Hg_x | solution (Me⁺= c_1)|| solution (Me⁺= c_2) | Me Hg_x

The potential of this cell will depend only on the relative activities of the metallic ions in the two solutions. If a_{Me} , the activity of the metallic ions, is known for one solution, it can be calculated

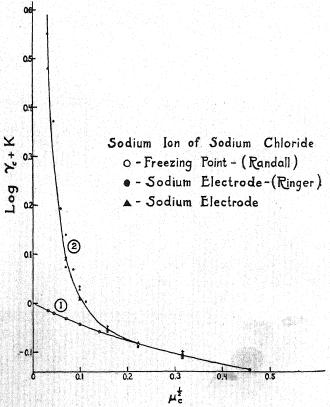


Fig. 25. Curves showing a comparison of the values of $\log \gamma_0 + K$ at corresponding ionic strengths for the sodium ion of sodium chloride in dilute solutions, as obtained from measurements of freezing point lowering (Curve 1) and from measurements with the sodium amalgam electrode (Curve 2).

(Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

for the other solution with the aid of the Nernst equation:

$$E = \frac{RT}{nF} \ln \frac{a_{\text{Me}^+}}{a'_{\text{Me}^+}} \tag{49}$$

It is probable that saturated KCl against solutions not exceeding about 0.1 molal show a constant diffusion potential. According to the above scheme liquid junction potentials will be cancelled out if the concentration of the solutions used does not exceed 0.1 molal.

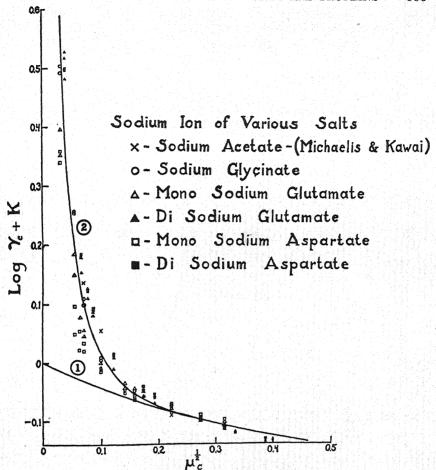


Fig. 26. Comparison of values for $\log \gamma_c + K$ for the sodium ion of certain sodium salts in dilute solution, with the typical curves (Nos. 1 and 2) for sodium chloride reproduced from Fig. 2, at corresponding ionic strengths.

(Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

The basis for the method of plotting the data rests on the rule stated by Lewis and Randall (28b) that the activity coefficient of any ion depends solely upon the total ionic strength of the solution. If the measured value of E is divided by 2.303 RT/nF, it will be proportional to $\log a_{\rm Me}^+$. Dividing $a_{\rm Me}^+$ by $c_{\rm Me}^+$, where $c_{\rm Me}^+$ equals the concentration of Me⁺ in moles per liter, and changing the sign of E, the expression,

$$\frac{-E}{2.303 \frac{RT}{nF}} -\log c_{\text{Me}} + = \log \gamma_c + K'$$
 (50)

for Me $^+$ is obtained, where K' is some constant depending on the type of calomel half-cell used, the uneliminated diffusion potential and the characteristic of the electrode depending chiefly on the

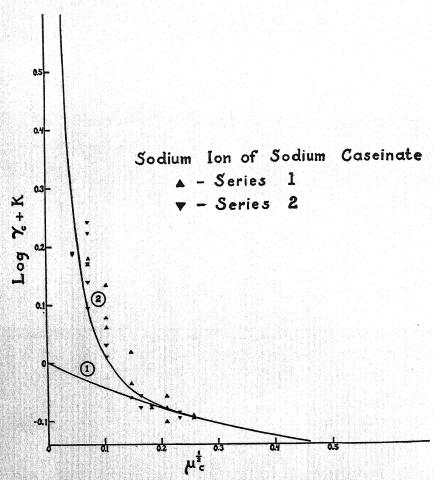


Fig. 27. Comparison of values for $\log \gamma_c + K$ for the sodium ion of sodium caseinate with the typical curves (Nos. 1 and 2) for sodium chloride reproduced from Fig. 2, at corresponding ionic strengths.

(Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

concentration of Me in the amalgam. γ_c applies to the sodium ion activity coefficient rather than to that of NaCl. Even though no absolute value can be assigned to γ for the sodium ion, it seems highly probable that it differs negligibly from γ for NaCl in a dilute solution of NaCl. At infinite dilution $\log \gamma_c$ is zero, since γ_c

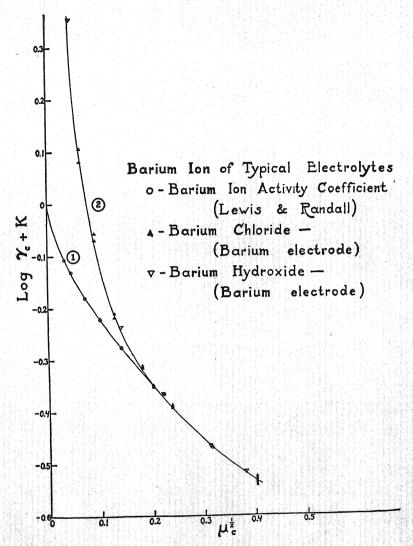


Fig. 28. Curves showing a comparison of the values of $\log \gamma_c + K$ for the barium ion of barium chloride and barium hydroxide in dilute solutions, as obtained from measurements with the barium amalgam electrode (Curve 2), with the activity coefficient values of Lewis and Randall for the barium ion (Curve 1), at corresponding ionic strengths.

(Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

becomes 1. Since the solutions were prepared in terms of moles per liter, the ionic strength, which is usually designated by μ , and, in the case of uni-univalent electrolytes, is equal to the molality, has been designated by μ_c .

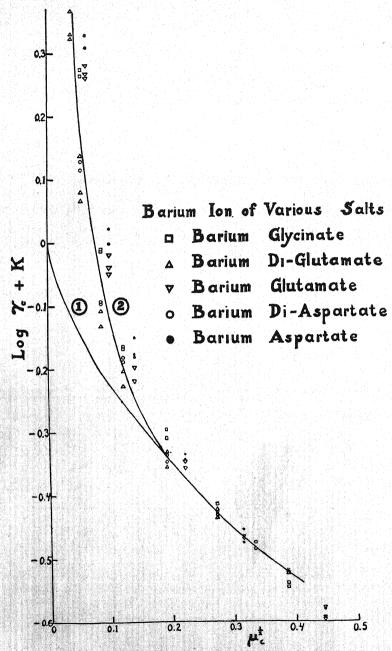


Fig. 29. Comparison of values for $\log \gamma_c + K$ for the barium ion of the barium salts of certain amino acids with the typical curves (Nos. 1 and 2) for the barium ion of strong electrolytes reproduced from Fig. 5, at corresponding ionic strengths. (Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

The data obtained by Kirk and Schmidt are graphically represented in Figs. 25 to 30. If the type of curve given in Fig. 25 is typical for the method used, and is dependent only on that and on the activity of the sodium ion, then it seemingly follows that any strong electrolyte which yields sodium ions should give this type of curve. This hypothesis is borne out by the data which were obtained with a number of amino acids and with sodium acetate and which are graphically represented in Fig. 26. Seemingly, then, these substances behave in solution as strong electrolytes.

In dealing with sodium caseinate one is handicapped by the fact that neither the charge on the protein nor its equivalent weight is definitely known. Cohn and Hendry (52) conclude that casein behaves as a dibasic acid. However, at the pH at which sodium ion activity measurements were carried out, all of the groups in the casein molecule which are dissociated are of about equal strength. Since it is probable that the total valence has little or no significance in determining the ionic strength of a casein solution when the pH is at or near the neutral point, it is perhaps logical to assume that this protein behaves as a univalent ion with respect to the ionic strength. This assumption has been made in plotting the data which are represented in Fig. 25. No other assumption regarding the valence of casein fits the curves so well. The conclusion of Stadie and Hawes (53) that the ionic strength valence is 1 is in line with the present assumption.

As seen from Fig. 27, the data which were obtained with sodium caseinate solutions fall fairly close to the sodium chloride curve. In this case it also appears that the sodium ions in a sodium caseinate solution have the same activity as in any strong electrolyte at the same ionic strength. The sodium salt of casein must be a typically strong electrolyte and consequently totally dissociated in the sense implied in recent theories of strong electrolytes (54). This view is in line with the conclusion which was drawn from transport data and presented earlier in this chapter. It also harmonizes with the work of Ferguson and Bacon (18) who conclude from the results of diffusion potential measurements that gelatin chloride is highly ionized. In theoretical treatments it is usually assumed that the activity coefficient of protein salts is unity. However, in the case of sodium chloride, this value is obtained only in extremely dilute solutions. Hence, as a first approximation, it is more logical to assume, in equal concentrations, that the activity coefficients of protein solutions such as sodium caseinate are approximately the same as that of sodium chloride.

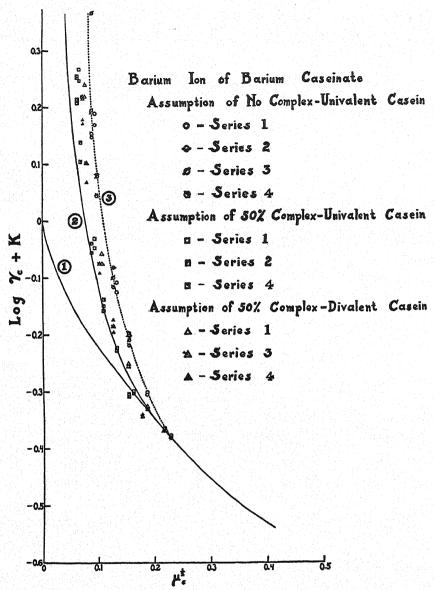


Fig. 30. Comparison of values for $\log \gamma_c + K$ for the barium ion of barium caseinate with the typical curves (Nos. 1 and 2) for the barium ion of strong electrolytes reproduced from Fig. 5, when certain assumptions are made.

(Kirk, P. L., and Schmidt, C. L. A., J. Biol. Chem., 76, 115 (1928).)

As seen from the data represented in Figs. 28 and 29, the behavior of the barium electrode in dilute inorganic barium salt solutions and in solutions of the several barium salts of amino acids is essentially similar to the behavior ascribed to the sodium electrode. It seems justifiable to conclude that the barium salts of these amino acids likewise behave as strong electrolytes. In plotting the data which are represented in Fig. 30, the assumption was made that (a) no barium casein complex ions are present, (b) 50 per cent complex-univalent casein ions are formed, and (c) 50 per cent complex-divalent casein ions are present in barium caseinate solutions. The method of plotting is not especially sensitive to the effect under consideration. However, the data are seemingly best interpreted on the assumption that casein plays the rôle of a univalent ion and that complex barium caseinate ions are present in solution. The data do not give a definite idea as to the magnitude of the complex formation.

16. HYDROGEN AND CHLORIDE ELECTRODES

The use of the hydrogen, quinhydrone, and glass electrodes for the purpose of determining the acid and base combining power of amino acids and proteins is too well known to warrant any discussion here. Reference to this subject is given in Chapter XI. A detailed discussion of these electrodes is given by Clark (55) and Hitchcock (56).

The application of the chloride electrode to measurements of the acid combining capacity of proteins deserves brief consideration. The combination of protein with chloride ion, as well as with hydrogen ion, may be determined with the aid of a cell, without liquid junction, of the type:

Ag, AgCl, HCl+protein, H2

The following discussion is taken from the work of Hitchcock (57). The E.M.F. of this cell at 30° is given by the equation,

$$E = E_0 - 0.06015 \log m_{\rm H} m_{\rm Cl} \gamma^2 \tag{51}$$

in which E = E.M.F. in volts after correction to unit hydrogen pressure, $E_0 = a$ constant depending on the nature of the electrodes and the temperature, m_H and m_{Cl} = the molalities of free H⁺ and Cl⁻, respectively, in the solution, and γ = the geometric mean activity coefficient of the ions of HCl in the solution.

If some of the ions of the HCl are combined with the protein, then $m_{\rm H}=m-gx$ and $m_{\rm Cl}=m-gy$ where m=molality of the total HCl, free and combined, g=protein concentration in gm. per 1000 gm. of water, and x and y=number of moles of H⁺ and Cl⁻,

respectively, combined with 1 gm. protein. Substituting the above terms and letting $E_0' = E_0 - 0.1203$ log γ , equation (51) becomes

$$E = E_0' - 0.06015 \log (m - gx) - (m - gy)$$
(52)

In order to solve for x and y it is necessary that the value for E_0 be known. The quantity E_0 should not be changed by the presence of protein while γ is probably changed. As a first approximation it may be assumed that γ and hence E_0 is unchanged by the protein. Using the value, 0.2310, which was obtained from measurements

Table XXXIII

Electromotive Force at 30° of the Cells Ag, AgCl, HCl+Gelatin, H2

m	g	E (observed)	E ₀ '	$E \ ({ m calculated})$	$\Delta extbf{\emph{E}}$	pH (approx.)
0.1000	50.3	0.3708	0.2306	0.3709	+0.0001	1.3
0.1000	70.4	0.3841	0.2304	0.3840	-0.0001	1.5
0.1000	90.5	0.4084	0.2302	0.4084	0	1.9

 $m = \text{moles HCl per 1000 gm. H}_2\text{O}.$

g = gm. dry gelatin per 1000 gm. H₂O.

E (observed) = E.M.F. in volts, corrected to 1 atmosphere dry H_2 .

 $E_0' = E + 0.1203$ log m, for cells containing HCl alone, of molality equal to m - qu ($E_0' = 0.2310$ for m = 0.1002).

 $E \text{ (calculated)} = E_0' - 0.06015 \log (m - gx)(m - gy).$

 $x = \text{moles H}^+ \text{ combined with 1 gm. gelatin} = 9.58 \times 10^{-4}$.

 $y = \text{moles Cl}^- \text{ combined with 1 gm. gelatin} = 2.0 \times 10^{-4}$.

 $\Delta E = E$ (calculated) -E (observed).

pH (approx.) = $-\log (m - gx)$.

(Hitchcock, D. I., J. Gen. Physiol., 15, 125 (1931).)

with 0.1002 molar HCl free from protein, the three equations resulting from the experiments which are given in Table XXXIII are represented graphically by assuming values for x, calculating y, and plotting y against x. The curves so obtained intersected at points corresponding to x = 9.6 to 9.7×10^{-4} and y = 1.6 to 1.8×10^{-4} . As a second approximation it is assumed that E_0 should be equal to the value obtained with pure HCl, not of molality m, but of molality m-gy. The difference m-gy represents some free HCl and some HCl of which only the H+ is bound to the protein. On the assumption that the ionic strength principle, as given by Lewis and Randall, applies to mixtures of HCl and an ionized protein hydrochloride, and, if the positive protein-hydrogen ion is assumed to have an effective valence of one in its effect on the ionic strength,

then the value of γ or E_0' for such a mixture should be the same as that for HCl of molality m-gy. For a second approximation y is assumed to be 1.7×10^{-4} and values of E_0' are read off from a plot of the values obtained for HCl in connection with an earlier study by Hitchcock (58) on edestin, values for E_0' being plotted against \sqrt{m} . A graphical solution of these equations yields curves which intersect at points whose coordinates average 9.6 and 2.0 $\times 10^{-4}$. A third approximation on the basis $y=2.0\times 10^{-4}$ gives intersections for which the average value of y is again 2.0×10^{-4} . The values of E_0' corresponding to m-gy, using this value for y, are given in the fourth column of Table XXXIII which is taken from Hitchcock's paper (57).

The data indicate that gelatin in 0.1 M HCl combines with a maximum of 9.58×10^{-4} equivalents of H⁺ and 2.0×10^{-4} equivalents of Cl⁻. In a similar manner Hitchcock found that 1 gm. of edestin (or edestan), when dissolved in 0.1 M HCl, combines with a maximum of 13.4×10^{-4} equivalents of H⁺ and 3.9×10^{-4} equivalents of Cl⁻.

17. POLAROGRAPH

The polarograph, which was developed by Heyrovský (59) has, in recent times, been used in studies appertaining to proteins and amino acids. It employs a mercury dropping electrode as the cathode and a mercury electrode of the second order as the anode. The course of the electrolysis is recorded automatically on photographic paper as current voltage curves. On the basis of these curves, both the qualitative and quantitative composition of solutions which contain electro-reducible compounds can be determined. When several such compounds are present, they can be distinguished from each other according to the differences in their characteristic reduction potentials, and their individual concentrations can be estimated from the values of the limiting current intensities which correspond to the horizontal portions of the curve. The current-voltage curves of solutions which contain iodoacetic acid show a distinct reduction which corresponds most probably to the reaction.

$ICH_2COOH + H^+ + 2e \rightarrow CH_3COOH + I^-$

The limiting current intensities of this reaction, measured in millimeters (see Fig. 31) are proportional to the concentration of iodoacetic acid. The proportionality constants expressed by the

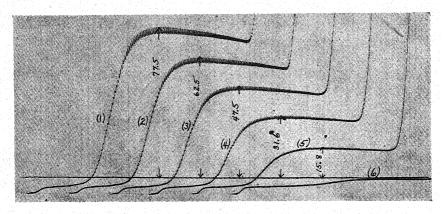


Fig. 31. Current-voltage curves (sensitivity of galvanometer 1/500).

No.

1 (9.92×10⁻³ N)

2 (7.92×10⁻³ N)

3 (5.95×10⁻³ N) sodium iodoacetate in 0.08 N sodium carbonate solution.

4 $(3.96 \times 10^{-3} \text{ N})$

5 $(2.00 \times 10^{-3} \text{ N})$

6 Atmospheric oxygen dissolved in 0.08 N sodium carbonate solution. (Brdička, R., J. Gen. Physiol., 19, 843 (1926).)

ratio, Limiting current/Conc. of iodoacetic acid, are given in Table XXXIV. Besides the reduction of the iodoacetate, the current-voltage curves include the reduction of the atmospheric

	BLE		

Curve Limiting current (mm. on scale)	1 77.5	$\begin{array}{c}2\\62.5\end{array}$	3 47.5	4 31.6	5 15.8
Concentration of iodoacetic acid (equiv's, ×103)	9.92	7.92	5.95	3.96	2.0
Ratio	7813	7891	7983	7980	7900

(Brdička, R., J. Gen. Physiol., 19, 843 (1936).)

oxygen which is present in the aqueous solution in about 10^{-3} equivalent concentration. This reduction takes place in two steps (Fig. 31, curve 6), the first corresponding to the reaction,

$$O_2+2 H_2O \rightarrow 2 H_2O_2$$

and the second to

$${\rm H_2O_2}{+2}~{\rm H^+} + 2e{\to}2~{\rm H_2O}$$

The current due to the oxygen reduction can be determined in the solution and subtracted from that of the corresponding solutions

which contained iodoacetate. Quantitative estimations of iodoacetic acid in buffer solutions can be carried out in several minutes with an accuracy of ± 1 per cent.

Brdička (60) has used this instrument in a study of the reaction which takes place between glycine and iodoacetic acid. He ob-

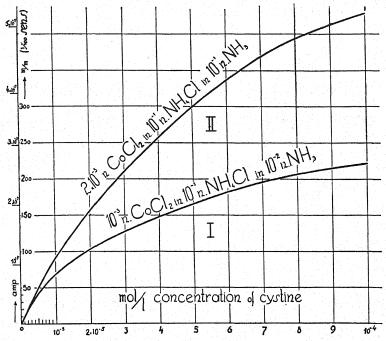


Fig. 32. Calibration curves obtained by means of a standard solution of cystine for determination of cystine-content in hydrolysates of proteins.

(Brdička, R., Czechoslovak Chem. Com., Collect. 5, No. 6, p. 238 (1933).)

tained evidence which indicates that (a) the reaction proceeds in two steps in which the hydrogens of the amino group are consecutively replaced by acetyl radicals, the velocity constants being in the ratio 2:1, (b) only the anionic form of glycine is able to react since the velocity constants at any pH are proportional to the concentration of glycine anion, and (c) the reaction is of the ionic type, showing a positive salt catalysis, which, according to Brönsted's hypothesis, involves the primary and the secondary salt effects. The fact that only the glycine anion is able to react is apparently due to the existence of an unbonded pair of electrons on the nitrogen in the NH₂ group. The NH₃+ group, however, in which these electrons are shared by H+, must, therefore, be inactive.

Brdička (61) has also used the polarograph for the estimation of cystine in protein hydrolysates. The method involves the estimation of the current-voltage curves which are obtained in solutions containing cystine, 10⁻³ N CoCl₂, and 0.1 N each of NH₄Cl and NH₃. Hydrogen is liberated at the dropping mercury cathode as

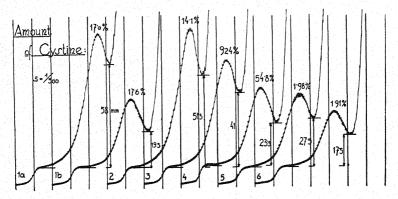


Fig. 33. 50 × dilute solution of 1 (a) 0.0342 gms of human hair A, 1 (b) 0.00075 gms of human hair A (in 1 cc), (2) 0.0341 gms of human hair E, (3) 0.0377 gms of wool, (4) 0.0031 gms of glutathione, (5) 0.1029 gms of blood-albumin, (6) 0.0597 gms of egg-albumin hydrolyzed with 10 cc. of 5 N HCl, electrolyzed in $2 \times 10^{+3}$ N CoCl₂ in 10^{-1} N NH₄Cl, in 10^{-1} N NH₃.

(Brdička, R., Czechoslovak Chem. Com., Collect. 5, No. 6, p. 238 (1933).)

the result of the catalytic effect of cystine. The height of the catalytic waves which are measured is used as a measure of the concentration of cystine or other thio-acids (see Figs. 32 and 33). Amounts of cystine as small as 0.012-0.24 mg. per 10 cc. of solution can be determined with an accuracy of ± 5 per cent.

18. ACTIVITY COEFFICIENTS OF AMINO ACIDS AND PROTEINS

The data which are obtained from hydrogen or other electrode measurements permit the calculation of the activity of a particular ion rather than its concentration although numerically the value for these two terms may be nearly identical and this is usually assumed so. In a strict sense the molality, m, is equal to the activity, a, divided by the activity coefficient, γ . The determination of the last quantity is based on freezing point, diffusion, vapor pressure, boiling point, and electromotive force measurements. The first three methods are best adapted to the estimation of activity coefficients

¹ This is approximately true in dilute solutions only.

of amino acids. In fact, the reported data have been obtained by the use of the first two methods almost entirely.

Freezing point determinations may be carried out conveniently by the procedure outlined by Adams (62). The essential features are (a) thorough mixing of the solution with a large amount of ice, (b) determination with a multiple junction thermoelement and sensitive potentiometer the difference between the equilibrium temperatures of the ice solution of amino acid and water, and

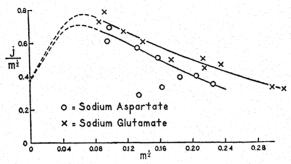


Fig. 34. Freezing point function for the monosodium salts of aspartic and glutamic acids.

(Hoskins, W. M., Randall, M., and Schmidt, C. L. A., J. Biol. Chem., 88, 215 (1930).)

(c) analysis of the equilibrium solution. The calculations (24) are made with the aid of the equation,

$$j = 1 - \frac{\theta}{\lambda m \nu} \tag{53}$$

where ν = the number of ion molecules which are formed by the dissociation of a molecule, λ = molal lowering of the freezing point at infinite dilution (1.858), m = the molality, and θ = freezing point lowering. The ratio between a given molal lowering and the molal lowering at infinite dilution is given by $\theta/\lambda m\nu$. In the case of the dicarboxylic amino acids the assumption may be made that the ionization of the second carboxyl group is negligible as compared with the first, hence ν = 2.

For the purposes of calculating γ , the activity coefficient, the following equation is used:

$$\log \gamma = -\frac{i}{2.3} - \frac{2}{2.3} \int_0^m \frac{j_2}{m^{1/2}} dm^{1/2}$$
 (54)

Values for the integral are obtained by plotting $j/m^{1/2}$ (the freezing

point function) against $m^{1/2}$ as shown in Fig. 34 and the area below the curve is integrated graphically. This is algebraically increased by an area which represents the difference between the measured curve and the theoretical curve ($\Delta j/m^{1/2}$) is plotted against $m^{1/2}$). The "overall" activity coefficients of aspartic acid and glutamic acid and their monosodium salts, which are given in Table XXXV, were calculated in accordance with the above ideas.

Hoskins, Randall and Schmidt (24) have also calculated the values for γ_u (see Table XXXVI), the activity coefficient of the

Table XXXV

Activity Coefficients of Aspartic and Glutamic Acids and Their

Monosodium Salts at 0°

m	Aspartic acid	γ
0.0025		0.202
		0.139
		0.111
		0.097
0.015		0.076
		0.065
0.025		0.057
0.030		0.051
	Glutamic acid	
0.0025		0.169
0.0050		0.116
		0.092
0.01		0.080
0.015	andra de la companya de la companya En esperiencia de la companya de la	0.064
0.020	andropolitika (h. 1921). 1945 - Albander Britania (h. 1944).	0.055
0.025		0.048
0.030		0.044
	osodium aspartate	
0.01		0.830
	e de la journal	0.777
0.03	ရက် (၂၈) ရက ကို သောကြောင်းကြုံးကြုံးကြုံးကြုံးကြုံးကြုံးကြုံးကြုံ	0.750
0.04		0.731
		0.720
0.06		0.711
	osodium glutamate	
0.01		0.817
0.02		0.760
0.03		0.726
		0.703
		0.686
0.06		0.673
그렇게 하다 하다 모양이 들었다고 하다고 않게 되었다.		

(Hoskins, W. M., Randall, M., and Schmidt, C. L. A.; J. Biol. Chem., 88, 215 (1930).)

Table XXXVI

Activity Coefficients of the Undissociated Part of Aspartic
and of Glutamic Acids

Substance analyzed	m_{u}	γ_u
Aspartic acid	0.01	0.606
	0.02	0.516
	0.03	0.471
Glutamic acid	0.01	0.702
	0.02	0.644
	0.03	0.615

(Hoskins, W. M., Randall, M., and Schmidt, C. L. A., J. Biol. Chem., 88, 215 (1930).)

undissociated parts of these amino acids. The values for γ_u were calculated with the aid of the following equations:

$$\log \gamma_u = -\frac{j_u}{2.303} - \frac{1}{2.303} \int_0^{m_u} \frac{j_u}{m_u} dm_u \tag{55}$$

where $j_u = 1 - \theta_u / \nu_u \lambda m_u$. The value for ν_u is taken as unity $(\nu_u = 0$ when m = 0) in calculating values for the freezing point function, j_u / m_u .

The activity data which are obtained from freezing point measurements apply only to the low temperature at which they were obtained. The activity at other temperatures may be obtained with the aid of the equation,

$$d \ln (a/a^0) d T = -(\overline{H} - \overline{H}^0/RT^2)$$

where a = activity. Values for $\overline{H} - \overline{H}^0$ for some of the amino acids are given in Chapter XV.

Activity coefficients for some of the amino acids, based on freezing point measurements, have been reported by Frankel (63), Lewis (64), Cann (65) and Scatchard and Prentiss (66). The data of the first three mentioned workers are expressed, respectively, by the following equations:

ln
$$\gamma = -0.1814$$
 ln $m - 0.2538$ (Frankel),
ln $\gamma = -0.1616$ ln $m - 0.2165$ (Lewis),
ln $\gamma = -0.0607$ ln $m - 0.0396$ (Cann).

Mehl and Schmidt (37) have recently obtained activity coefficient data which are based on diffusion measurements of amino acids. They made use of the equation,

$$D = \left(\frac{\Omega}{c}\right) RT(1 + c \,\partial \log \gamma / \partial c) \tag{57}$$

where D = diffusion coefficient, γ = activity coefficient, c = concentration, and Ω/c = the "mobility" of the molecular or ionic species. It is probably constant for a given non-electrolyte, at least in moderate concentrations. There are two ways in which the activity coefficient a/m or γ_m might be calculated from equation (57). The more direct way to make the calculation would be to use the

TABLE XXXVII(a)

Activity Coefficients

Concen- tration		erature 0°			erature 1°	Fre	Freezing Point Data		
(moles/1000 gm. H ₂ O)	γm	$\gamma_M(M/m)$	Υm	$\gamma_M(M/m)$	Υm	$\gamma_M(M/m)$	Lewis	Scatchard and Prentiss	Frankel
				(a)	Glycine				
0	1.000	1.000		()	1.000	1.000	1.000	1.000	1.000
0.2	0.970	0.974			0.958	0.964	0.962	0.957	0.942
0.4	0.939	0.953			0.916	0.925	0.927	0.918	0.890
0.6	0.910	0.930			0.879	0.892	0.893	0.883	0.847
0.8	0.882	0.908			0.847	0.863	0.864	0.853	0.810
1.0	0.857	0.888			0.818	0.838	0.840	0.826	0.779
1.2	0.833	0.870			0.800	1	0.817	0.802	0.752
1.4	0.811	0.851			0.000	, 0.021	0.011	0.002	002
1.7	0.511	0.001		1					
				(b) d,	l-Alanin	e			
0	1.000	1.000			1.000	1.000			
0.2	0.985	0.990			0.977	0.988			
0.4	0.948	0.976			0.948	0.970			
0.6	0.920	0.957			0.915	0.946			
0.8	0.892	0.933			0.885	0.919			
1.0	0.863	0.911			0.855	0.891			
1.2	0.840	0.900							
	1 000	1 1 000		(c) a	, l-Valine				
0	1.000	1.000			1.000				
0.1 0.2	0.975	0.984			0.961	0.970			
	0.952	0.967			0.926	0.942			
0.3	0.929	0.952			0.897	0.918			
0.4	0.907	0.936			0.874	0.901			
0.5	0.887	0.920		1	0.856	0.889			
				(d) l-A	ı Asparagir	ie .			
0	1.000	1.000	1.000	1.000	1				
0.05	0.977	0.980	0.954	0.956					
0.10	0.955	0.961	0.911	0.916					
0.15	0.933	0.943	0.870	0.877					F 15 (1)
0.20	0.912	0.926		1877					
0.25	0.892	0.908							
0	1.000	1.000		(e) d	, l-Prolin	е			
0.1	0.978	0.989			100				
0.1	0.957	0.939			100				
0.2	0.936	0.970							Production (
0.5	0.896	0.950				g turk kerel			
0.7	0.858	0.930							
7.1	0.000	0.331						All Landing	La salar da

		TABLE X	XXVII(b)			
Activity	Coefficients	of Glycine	Reported by	Smith	and Smith	r

m_A	ϕ_{25}°	ϕ_0°	γ25°	γo°
0.1	0.990	0.989	0.980	0.978
0.2	0.981	0.978	0.962	0.957
0.3	0.973	0.968	0.946	0.937
0.4	0.965	0.958	0.930	0.918
0.5	0.957	0.949	0.915	0.900
0.7	0.944	0.932	0.888	0.868
1.0	0.928	0.910	0.854	0.826
1.2	0.921	0.898	0.838	0.802
1.5	0.913	0.884	0.816	0.771
1.7	0.908	0.876	0.801	0.754
2.0	0.903	0.869	0.786	0.733
2.5	0.894		0.760	
3.0	0.888		0.741	
3.3	0.885		0.729	

Data at 0° are from Scatchard and Prentiss. Those at 25° are from Smith and Smith. The data of Smith and Smith were obtained by the isopiestic method.

(Smith, E. R. B., and Smith, P. K., J. Biol. Chem., 117, 209 (1937). This paper appeared since this chapter was written. See also J. Biol. Chem., 119, Proc. XCI, (1937), and CX (1938).)

values obtained for D_m . Assuming Ω/c to remain constant, this method would assume the constancy of Ω/m . Values for γ_m might also be obtained by using the value for D_M in equation (57) and multiplying the values of γ_M thus obtained by M/m. In this case it would be assumed that Ω/M is a constant. It is evident that both Ω/m and Ω/M cannot be constants. It is possible that neither quantity is a constant. The assumption of the constancy of Ω/c is the weakest point in the calculation of activity coefficients from diffusion data. The validity of the assumption can be tested by comparing values of the activity coefficients which are calculated from diffusion data with available data which have been obtained in other ways. Such a comparison is made in Table XXXVII.

Although the values of $\gamma_M(M/m)$ for glycine appear to be in better agreement with the freezing point data of Scatchard and Prentiss (66) and of Lewis (64) than the values of γ_m , the differences between $\gamma_M(M/m)$ and γ_m are not much greater than the possible errors to which they are subject. It is not possible to decide from the values for glycine whether it is more nearly correct to consider Ω/m or Ω/M a constant. With the exception of the data which Frankel (63) has reported for alanine and valine, no other cryoscopic data appear to be available for purposes of making further comparisons. The values for these amino acids appear to be less satisfactory than those which he has reported for glycine. The data for the boiling points of asparagine solutions which Frankel (63) has reported cannot be used for purposes of checking

quantitatively the values of the activity coefficients which have been obtained from diffusion data. In a qualitative way the agreement is satisfactory. Considerably more work relating to activity coefficients of amino acids will have to be carried out before the subject can be considered to be in a satisfactory state.

Osmotic pressure measurements have been used for purposes

TABLE XXXVIII

The Activity Coefficients of a Salt of Hemoglobin and Its Ions

Conditions: Temperature, 0°; pressure, 760 mm.; composition of "solvent," 0.10 mole of KCl, 0.0613 mole of Na₂HPO₄, 0.00533 mole of KH₂PO₄ per liter. Composition of protein salt, Hb(Na+K)_{8.5}. Valence of protein ion, $n_p = -8.5$. The activity coefficients of the sodium and potassium ions are approximately equal to 0.7.

Hb. per liter of solvent, mole, m_p .	Act. of protein salt, $\dagger a_{ps}$.	Act. coeff. of salt, f_{ps} .	Act. coeff. of ion, f_p .
0.0002	0.0002	1.05	1.03
0.0010	0.0014	1.40	1.3
0.0020	0.0040	2.0	1.7
0.0030	0.0090	3.0	2.3
0.0040	0.0200	5.0	3.6

[†] The determination of a_{ps} by the evaluation of the integral in the above formula may be facilitated by application of the empirical formulas discussed in Proc. Roy. Soc., London, 120A, 573 (1928). In dilute solutions the formula $p(v_s-b)=RT$ can be applied and by integration RT in $a_{ps}=fv_sdp=RT$ in p+bp. Over the range of pressures from 12 to 120 mm., a formula with two empirical constants can be applied, $p(v_s-107.5)=1.09$ RT.

(Adair, G. S., J. Amer. Chem. Soc., 51, 696 (1929).)

of determining the activity coefficients of protein solutions. In studying certain salts of hemoglobin, Adair (67) made the assumption that the average valence of the protein ions is -8.5. His calculations were made with the aid of the equation,

$$RT \ln f_{ps} m_p = RT \ln a_{ps} = \int v_s dp + \text{constant}$$
 (58)

where m = gram moles of protein salt per liter of solvent, and $f_{ps} = a_{ps}/m_p$ (a coefficient which approaches unity at low protein concentrations). The data are given in Table XXXVIII.

19. VALENCE OF PROTEIN IONS

Reference has been made to the subject of the valence of protein ions. In some of the calculations in which this quantity enters values have been assumed. Adair and Adair (68) have attempted to obtain values for several proteins from membrane potential measurements. They employed the equation,

$$n_p = 0.00425 \ MJ(E/C\gamma)_0$$
 (59)

where n_p = mean valence of the protein ions, M = molecular weight of the protein, J = sum of the concentrations of the ions in the dialysate multiplied by the square root of the valences, and $(E/C\gamma)_0$ = limiting values of the ratio at $C\gamma - O$, as determined by extrapolation. A rough value for edestin was found to be 24 on the assumption that the activity coefficient of H⁺ is the same as that of the Cl⁻ ions. The approximate mean value for serum albumin was reported to be -25.4.

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CHAPTER XIII

COMBINATION OF AMINO ACIDS AND PROTEINS WITH ACIDS, BASES, HEAVY METALS, AND OTHER COMPOUNDS

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1. COMBINATION WITH ACIDS AND BASES

(1) Acid and Base Titration Curves

In 1898 Bugarszky and Liebermann (1) demonstrated, by means of electromotive force measurements, that egg albumin combines with both acids and bases. A year later, Osborne (2), using a colorimetric method, established the fact that various amounts of acids were required to neutralize the basic groups in a number of proteins. The method employed by the first mentioned workers deserves consideration. They determined the electromotive force between two hydrogen electrodes, one of which was immersed in 0.05 N hydrochloric acid, and the other of which was immersed in the same amount of this acid to which varying amounts of the protein were added. The amount of acid which was combined with the protein was calculated by means of the Nernst equation,

$$E = \frac{RT}{nF} \ln \frac{C_2}{C_1} \tag{1}$$

where C_2 =concentration of H⁺ in the acid to which protein had been added, and C_1 =concentration of H⁺ in the solution of HCl. Cohn (3) has recalculated Bugarszky and Liebermann's data. The recalculated data are given in Table I. It is evident that in the presence of an excess of hydrochloric acid, egg albumin is saturated with the acid. The maximum acid-combining capacity of this protein for hydrochloric acid is about 80×10^{-5} moles.

The acid- and base-combining capacity of proteins is now generally determined with the aid of one of the various types of electrodes (hydrogen, quinhydrone, or glass). The pH of the protein-containing solution is determined on addition of varying amounts

of acid or base. The calomel cell is used as the reference electrode. The technique of obtaining the titration curve of a protein is essentially similar to that used for amino acids (see Chapter XI).

Measurement of pH yields the activity and not the concentration of hydrogen ions. In order that the latter quantity may be de-

Table I

Acid-Combining Capacity of Egg Albumin Calculated from the Results of Bugarszky and Liebermann

Egg albumin in system	HCl in system	HCl free	HCl combined	HCl combined with 10 gms. egg albumin
p	a	b	a-b	a-b/p
gms.	N	N	N	N
6.4 3.2 1.6 0.8 0.4	0.05 0.05 0.05 0.05 0.05	0.0027 0.0262 0.0373 0.0436 0.0468	0.0473 0.0238 0.0127 0.0064 0.0032	0.0074 0.0074 0.0079 0.0080 0.0080

(Cohn, E. J., Physiol. Rev., 5, 349 (1925).)

termined, the activity coefficient, γ , of the hydrogen ions must be known, as is indicated by the equation,

$$pH = \log \frac{1}{\gamma(C_{\rm H}^+)} \tag{2}$$

The value for γ is influenced by the concentration of hydrogen ions, by the concentration, the valence, and the dimensions of all of the ions which are present in the solution, and by the dielectric constant of the solution. Uncertainty as to the valence of protein ions has made it impossible to take into account the influence of a particular protein ion on the value for γ (4).

The electrochemical method which was used by Bugarszky and Liebermann (1) and by Robertson (5), with certain refinements such as the use of the calomel cell as reference electrode, is perhaps the easiest way in which the acid- or base-combining capacities of proteins in aqueous solution may be determined. There is, however, the uncertainty of the magnitude of the potential of the liquid junction. In this method the influence of the anion of the

acid or the cation of the base neutralized by the protein is taken into account by the assumption, which is based on the ionic strength principle, that the activity of hydrogen or hydroxyl ions in the protein-containing solution is the same as that in a solution of the acid or base of equal ionic strength. In this assumption the influence of the protein ion is neglected. In dilute solution this factor is probably small. For most purposes it is convenient to assume that the values of the concentrations of hydrogen or hydroxyl ions are the same as those of the activities. In the case of aspartic and glutamic acids it has been shown by Miyamoto and Schmidt (6) that no appreciable error results from a similar assumption.

Rasmussen and Linderström-Lang (7) have made calculations of the activity coefficient of the hydrogen ion in solutions of clupein. They assumed that the ionic strength is the same at different stages in the titration of this protein and hence the value of γ , the activity coefficient, was taken as a constant. The value of γ was calculated with the aid of the equation,

$$K_{B} = \frac{aH^{+}\left(C_{\text{Prot.}} - C_{\text{HCl}} - \frac{aH^{+}}{\gamma}\right)}{C_{\text{HCl}} - \frac{aH^{+}}{\gamma}}$$
(3)

where C = concentration, $aH^+ = \text{activity}$ of the hydrogen ion, and K_B = the basic dissociation constant of the protein. Three connected pairs of values are necessary to determine K_B , $C_{\text{Prot.}}$, and γ from the above equation. Values for γ ranged from 0.78 to 0.91 for different preparations of clupein. These values cannot necessarily be taken as having real significance. They may represent a correction for the reduction of the activity of the hydrogen ion at the given ionic strength as well as a correction for the diffusion potential. On the other hand, they may indicate that the contribution of this protein to the ionic strength is much less than would be predicted for an ion of such high valency. It should be borne in mind that the charge on this protein ion has the same sign as that on the hydrogen ion and that the charges are distributed along the entire length of the protein molecule. These factors may reduce considerably the interactions of these protein ions and the hydrogen ions.

The above discussion indicates some of the uncertainties which

must be borne in mind when attempts are made to determine activity coefficients of hydrogen ions in protein solutions. Measurements of the acid- or base-combining capacities of proteins which have been carried out in strongly acidic or alkaline solutions may, at times, be in error due to incipient hydrolysis of the protein,

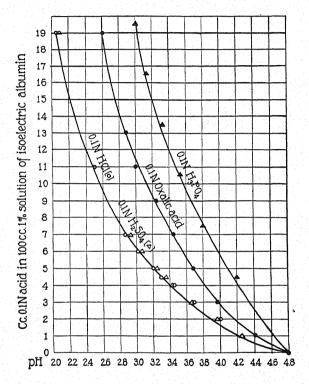


Fig. 1. The ordinates represent the number of cubic centimeters of 0.1 N HCl, $\rm H_2SO_4$, oxalic, and phosphoric acids required to bring 1 gm. of isoelectric crystalline egg albumin to the pH indicated on the axis of abscissae. Enough $\rm H_2O$ was added to bring the albumin and acid to a volume of 100 cc. For the same pH, the ordinates for HCl, $\rm H_2SO_4$, and phosphoric acid are approximately 1:1:3. The ratio of HCl to oxalic acid is a little less than 1:2 when pH is >3.0.

(Loeb, J., J. Gen. Physiol., 3, 85 (1920-21); Proteins and the Theory of Colloidal Behavior, 2d ed., New York and London, 1924, p. 53.)

hydrolysis of the acid amide group, or the opening of internal anhydrides, if these be present.

The combination between protein and acid or base is a stoichiometric one. This is nicely illustrated by Fig. 1. The curves for hydrochloric and sulfuric acid are identical, indicating that the combination between protein and these acids takes place in equiva-

lent proportions. Phosphoric acid combines with egg albumin in molecular proportions. The anion of albumin phosphate is therefore the monovalent ion, H_2PO_4 . When the pH is below 3.2 the values

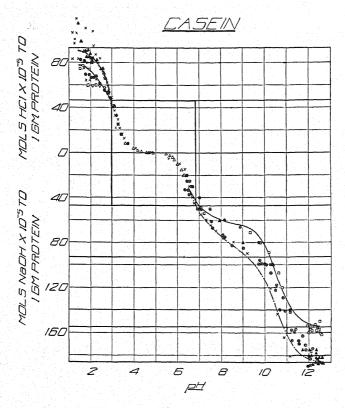


Fig. 2

Tourset and and the second and the s		Symbol	
Investigators		Modified	
Robertson	casein	casein ⊗	
Loeb			
Greenberg and Schmidt	0		
Hoffman and Gortner		×	
Hitchcock	∇		
Cohn and Hendry			
Cohn and Berggren	_	•	
		· · · · · · · · · · · · · · · · · · ·	

Robertson employed potassium hydroxide in his experiments.

Casein passes into solution when one gram is combined with approximately 47×10^{-5} moles of either sodium hydroxide or hydrochloric acid. The acid- and base-combining curves of normal and modified casein have been constructed on the basis of the following dissociation constants; pK = 3.00, 7.10, 8.46, and 10.55. (See Cohn, E. J., *Proc. XI Int. Physiol. Congress*, Edinburgh, 1923.)

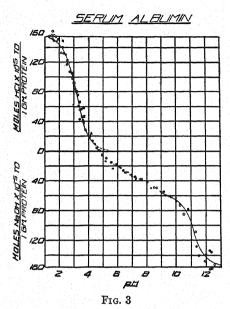
(Cohn, E. J., Physiol. Rev., 5, 349 (1935).)

for oxalic acid are almost, but not quite, twice as great as those for the strong acids, indicating that, for these pH values, oxalic acid combines with the protein in molecular rather than in equivalent proportions. The differences in the three titration curves are due to differences in the dissociation constants of the acids with which the protein is titrated, i.e., oxalic and phosphoric acids each yielding only one hydrogen ion. Similar differences in titration curves are obtained when a protein is titrated with bases whose dissociation constants differ from each other.

A great many measurements of the acid- and base-combining capacities of proteins have been carried out. The results which have been reported are not always uniform. This may, in certain instances, be due to differences in the purity of the protein preparations which were used. Fig. 2, which is taken from Cohn's review (3), is illustrative of the differences in the results which have been reported by various investigators. These differences probably can, in part, be ascribed to the difficulty of obtaining uniform preparations of casein. In the case of serum albumin (see Fig. 3) the results which different workers have reported are more uniform. In Figs. 2 and 3 equal weights of the proteins have been used. The acid- and base-combining capacities of various proteins are given in Table VIII of this chapter. According to Booth (8), heat denaturation does not change the acid- or base-combining capacity of proteins.

The slope of the titration curve at any point gives the rate of combination and therefore the relative number of groups dissociating in the protein molecule. The slopes, when plotted graphically, represent, as a first approximation, the number and strength of the dissociating groups in the uncombined protein molecules. As increasing amounts of acid or base are added, the rate of combination increases rapidly. The average rate of combination throughout this range may be estimated roughly from the slope of a diagonal drawn between the points at which solution takes place on addition of acid or alkali. The greater the dimensions of the rectangles, the greater the amount of acid or base which is required to dissolve the protein. The greater the horizontal dimensions, the greater is the difference in strength between the acidic and basic groups. When uncombined proteins are compared, the slopes of the tangents drawn to their titration curves at the isoelectric points are steeper for albumins than for the globulins. The tangents for the globulins are steeper than those for the glutelins and the prolamins. In

general, the slope is smallest for proteins which are insoluble in water at their isoelectric points; steeper for proteins which are



Investigators	Symbol
D'Agostino and Quagliariello	. Δ
Manabe and Matula	•
Pauli and Hirschfeld	. 🛦
Pauli and Odèn	. 📕
Hastings	. ×
Cohn and Berggren	. 00

D'Agostino and Quagliariello's preparation had a pH of 6.89. It has therefore been assumed that it contained 32×10^{-5} moles of sodium hydroxide per gram. From Pauli and Odèn's measurements 10×10^{-5} moles of acid were subtracted, in order that they might better conform with the other measurements made in Pauli's and in other laboratories. Pauli's measurements, demonstrating variation with time, have not been included, although they are of the same order.

The curve describing the combination with acid has been constructed on the basis of a single constant, pK=3.16. Combination at the more alkaline reactions can also be described on the basis of a single constant, pK=11.10. So many constants would be required to describe the continuous combination in the intervening space that they have not been calculated.

(Cohn, E. J., Physiol. Rev., 5, 349 (1925).)

slightly soluble; and steepest for those which are relatively soluble in the isoelectric condition. The amphoteric constants of a protein appear to be greater the smaller the molecular weight. Cohn (9) has pointed out that the dissociation of proteins determines those aspects of their behavior upon which their classification depends. Different amounts of acid or base are required by different proteins when they pass into solution. Values for a number of proteins are given in Table II.

Table II

The Slopes of the Titration Curves of Certain Proteins in the Neighborhood of Their Isoelectric Points

Protein	Slope of t titration isoelectr	_	Base required to dissolve protein		Acid required to dissolve protein	
	the state of the s	ation per unit				
	moles per gm. ×10 ⁵	moles per mole	moles per gm.×10 ⁵		moles per gm. ×105	moles per mole
Serum albumin	28	12.6				
Egg albumin	22	7.4				
Gelatin	18					
Hemoglobin	12	6.0				
Edestin			7	4	14	8
Serum globulin	5	4.0	7	6	14	12
Casein	3	5.8	47	90	47	90

(Cohn, E. J., Physiol. Rev., 5, 349 (1925).)

Rasmussen and Linderström-Lang (7) have used titration data for purposes of calculating the molecular weights of clupein preparations. Using the values for C_{Prot} obtained by means of equation (3) and substituting in the equation,

$$M = \frac{a \times 1000}{C_{\text{Prot.}}} \tag{4}$$

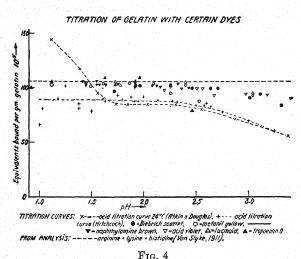
where M= molecular weight and a= grams of protein per unit volume of solution, the molecular weights of various clupein preparations ranged from 3600 to 4400. The molecular weights obtained from acetone titration data range from 3600 to 4300, and the range from alcoholic titration data is from 3700 to 4100. The molecular weight corresponds to 19 to 20 arginine and 7 to 8 monoamino residues. It is very probable that clupein preparations are mixtures of various clupein molecules.

The acid- and base-combining capacity of proteins has also been determined by use of the interferometer. Thomas and Mayer (10) have reported a break in the titration curve obtained by plotting the amount of acid added against the interferometer readings. This

occurred when 8.97 cc. of 0.1 N acid were added to 1 gm. of gelatin. The equivalent weight of the protein from these data is about 1100. Further discussion of this subject is given in Chapter X.

(2) Combination with Dyes

The estimation of the acid- or base-combining capacities of proteins by means of titration curves involves considerable error. The end-point of the titration is not sharp. The value obtained from a blank titration must be subtracted from the value which is obtained on titrating the protein. In regions of high acidity or alkalinity the change in pH on further additions of acid or alkali is so small that it becomes difficult to estimate accurately the value



(Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., J. Biol. Chem., 72, 707 (1927).)

of the blank. Moreover, at best, the titration represents an equilibrium between the protein salt and the excess of hydrogen or hydroxyl ions. A method which is free from these objections has been employed by Chapman, Greenberg, and Schmidt (11), and by Rawlins and Schmidt (12) for estimating the acid- and base-combining capacities of proteins. It consists in adding an excess of acidic or basic dye to solutions of a protein at varying pH values. Solid protein, such as gelatin granules, cannot be used so conveniently due to the extended period of time which is required to attain equilibrium. On addition of dye to the protein solution a precipitate is formed which can be removed by filtration With

certain dyes the end-point of the titration is indicated by the formation of a colorless zone, the total amount of dye added being precipitated in the protein-dye compound. In other cases the excess of dye in the filtrate can be estimated either colorimetrically or by titration with another dye (in the case of an acidic dye it is titrated with a basic dye and vice versa), allowing the dyes to be their own indicators. The compound formed by the union of such an acidic dye with a basic dye is usually insoluble. The pH of the filtrate is also determined. The data are plotted as a titration curve. Fig. 4 was obtained when gelatin was titrated with a number of acidic dyes.

Gelatin exists in the isoelectric condition at pH 4.7. Atkin and Douglas (15) extrapolated the acid and base titration curves of gelatin beyond the isoelectric point. It appears that gelatin is appreciably dissociated as a base to pH 5.8 to 6.0. A precipitate can be obtained by treating gelatin with Biebrich scarlet at pH 5.5 and with naphthylamine brown at pH 4.8. Grollman (16) found that phenol red combines with gelatin at pH 5.4. Loeb (17) noted that, at the isoelectric point, and even on the alkaline side of the isoelectric point, all of his gelatin granules, when treated with acidic dyes, such as fuchsin, did not give off their stain.

The data of a number of workers have indicated that the amount of acid with which a protein can combine can be correlated with the content of the basic amino acids (13). It appears that, in the native unsplit protein, the ϵ -amino group of lysine, the guanidine group of arginine, and the imino group of histidine are free. In the calculations which are presented in Table III the assumption has

Table III
Combining Capacity of Gelatin and Deaminized Gelatin

	Gelatin	Deaminized gelatin	Difference due to re- moval of the epsilon group of lysine
	Equivalents	Equivalents	Equivalents
발표 이 회사를 보고 말라고 있습니다. 그리고 그리고 하고 있다고 있다.	per gm.	per gm.	per gm.
			0 000445
Acid-combining capacity (Hitchcock)	0.000890	0.000445	0.000445
	0.000890 0.001066	0.000445 0.000661	0.000445
Acid-combining capacity (Hitchcock) Arginine +lysine + histidine (Van Slyke) Dye-combining ca- (Biebrich scarlet			

⁽Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., J. Biol. Chem., 72, 707 (1927).)

been made that at pH 1.0 the free basic groups of the three basic amino acids are completely dissociated. While this assumption is probably not strictly correct, the error involved is small, and for approximate calculations can be neglected. The data which are presented in Table III indicate that, at pH 1.0, the correlation between the dye-combining capacity of gelatin and the content of the basic amino acids is even better than that which is obtained when the value for the acid-combining capacity, obtained from titration data, is used. Deamination of gelatin, which is carried out by treating it with nitrous acid at a low temperature, reduces its combining capacity for acids and for acidic dyes by an amount which is approximately equivalent to the content of lysine (14). This experiment shows that it is possible to change the acidcombining capacity of proteins by chemical procedures. It is likewise possible to increase the base-combining capacity of proteins by converting the acid amide groups to carboxyl groups. This can be done by mild hydrolysis.

On the assumption that the acid-combining capacity of proteins is due to the free basic groups of arginine, lysine, and histidine, it is probable that, at pH 1.0, all of the groups in proteins are nearly completely ionized. Acidic dyes will unite with the basic radicles of the protein. If the protein-dye compound is sufficiently insoluble a precipitate is formed. At pH 2.0 or less, proteins which form insoluble compounds with acidic dyes combine with the maximum amount of dye. It appears desirable to ascertain how insoluble the protein-dye compound must be in order to unite with all of the basic radicals of the protein. The valence of protein ions is not known. However, it may be assumed that proteins acting as bases ionize as if they were monoacidic bases. The equation,

$$POH = P^+ + OH^-$$

where POH = protein base and P+=protein ion, may be used to represent the ionization of a protein as a base. Similarly,

$$P^++D^-=PD$$

where $D^-=$ an acid dye ion, and PD=protein-dye compound. Then

$$({\bf P}^+)({\bf D}^-) = K_s$$

will represent the solubility relations of the protein-dye precipitate.

If the protein is treated as if it were a weak base, then

$$\frac{(P^+)(OH^-)}{(POH)} = k_b$$

Letting S = total amount of protein in solution, $S - P^+ = POH$. It follows that

$$(OH^{-}) = k_{b} \frac{(S - P^{+})}{P^{+}}$$

$$\frac{(OH^{-})}{k_{b}} = \frac{S}{P^{+}} - 1$$

$$P^{+} = \frac{S}{\left(\frac{OH^{-}}{k_{b}} + 1\right)}$$
(5)

From the solubility product

$$P^{+} = \frac{K_s}{D^{-}} \tag{6}$$

Combining equations (5) and (6)

$$\frac{K_s}{D^-} = \frac{S}{\left(\frac{OH^-}{k_b} + 1\right)}$$

$$K_s = \frac{(S)(D^-)}{\left(\frac{OH^-}{k_b} + 1\right)}$$
(7)

Theoretically, equation (7) is not strictly correct due to the unknown valency of the protein ions. However, it may still be used as a model for the purpose of studying the solubility relations of protein-dye compounds. From equation (7) it is possible to calculate the greatest solubility which PD can have in order to be completely precipitated at pH 2.0 if k_b is taken as the dissociation constant of the weakest basic group, viz., the second basic group of histidine (5×10^{-13}) . With gelatin at pH 2.0, an end-point was obtained with Biebrich scarlet in which the dye concentration was estimated as being less than 7×10^{-6} equivalent per liter, and the concentration of gelatin was probably less than 0.001 gm. per $100 \text{ cc. or } 1 \times 10^{-5}$ equivalent of gelatin per liter. If the assumption

is made that the dye is totally dissociated at pH 2.0, then 7×10^{-6} equivalent per liter = D⁻. This is probably true since the concentration of dye is low. Dyes which contain a sulfonic acid radical are quite strong acids. By substituting these values in the formula it is possible to estimate what the greatest possible value of K_* has to be in order for this end-point to be a true one. Substituting values in equation (7),

$$K_s = \frac{(0.00001)(0.000007)}{\left(\frac{10^{-12}}{5 \times 10^{-13}} + 1\right)} = 2.3 \times 10^{-11}$$

This calculation, although rough, indicates that the value for K_s must be 2.3×10^{-11} or less, in order that at the end-point obtained (at pH 2.0) the dye will completely precipitate any base

Table IV
Solubility Products of Gelatin-Dye Compounds

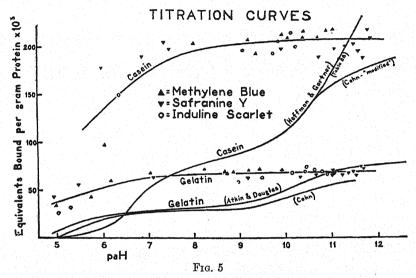
Dye	Conc. of gelatin ×106	Conc. of dye ×10 ⁶	Conc. (OH ⁻) ×10 ¹¹	pН	K.×1012
	Equiva- lents per liter	Equiva- lents per liter			
Naphthylamine brown	33	78	69.3	4.84	1.85
Naphthylamine brown	26	50	10.7	4.02	5.95
Biebrich scarlet Biebrich scarlet	21 77	33 105	3.88 41.6	3.58 4.62	9.10 9.90
Biebrich scarlet	130	380	329.0	5.51	7.65

(Chapman, L. M., Greenberg, D. M., and Schmidt, C. L. A., J. Biol. Chem., 72, 707 (1927).)

whose dissociation constant is 5×10^{-13} . Although this calculation is only approximate, it is probable that the value for K_s , in the case of gelatin-Biebrich scarlet and gelatin-naphthylamine brown, is about 2×10^{-11} or less. A check on this estimation was made by determining the concentration of gelatin and dye at a pH at which the gelatin-dye compound is soluble enough to exist in sufficient quantities to be analyzed quantitatively. In the calculations, the equivalence of gelatin is considered as 0.001 per gm. as an approximation, since titration data give about that value as the acid-combining capacity of this protein.

The data which are given in Table IV indicate that not only are the solubility product values in all cases less than the value which the rough estimates require them to be, but for each dye they agree as well as the large error involved in determining low concentrations of dye and gelatin will permit.

The capacity of proteins for combining with bases is due to the presence of free acidic groups (4, 13, 18). These are: the free carboxyl groups of aspartic, glutamic, and β -hydroxyglutamic



(Rawlins, L. M. C., and Schmidt, C. L. A., J. Biol. Chem. 82, 709 (1929).)

acid, the hydroxy group which is present in the phenyl ring of tyrosine, and the free acidic groups (in the case of phosphoproteins) of phosphoric acid. The free carboxyl groups may be estimated from the content of the dicarboxylic amino acids which are present in the protein molecule less those which are present in the form of acid amides (-CONH₂). The acid amide groups can be estimated on the basis of the ammonia which is obtained on hydrolysis of the protein.

The data which were obtained when certain proteins were treated, respectively, with methylene blue, safranine Y, and induline scarlet, and which are graphically represented in Figs. 5 and 6, indicate that, at pH 11.8, there is a close correlation between the amounts of the basic dyes which are combined with the proteins and the amounts of acid which are similarly combined. Thus, gelatin combines with 70×10^{-5} equivalents of dye. Simms' data

(18) for the base-combining capacity of gelatin agree with this value. On the basis of the content of dicarboxylic amino acids (the content of tyrosine is negligible) less the number of carboxyl groups which are present as acid amides, the expected value is 42×10^{-5} . It is possible that the difference between the experimental and the calculated value is due to hydrolysis of the acid amide groups. At pH 12.0, casein combines with 210×10^{-5} equivalents of dye. From acid titration data, values ranging between 160

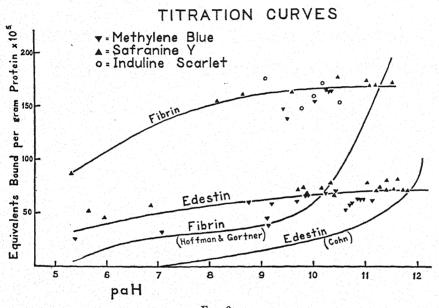


Fig. 6 (Rawlins, L. M. C., and Schmidt, C. L. A., J. Biol. Chem., 82, 709 (1929).)

 $\times 10^{-5}$ and 183×10^{-5} have been reported. The value calculated from the content of the free carboxyl and hydroxyphenyl groups is 158×10^{-5} equivalents per gram. It is possible that a certain portion of the acid amide groups was hydrolyzed. At pH 11.5, edestin combines with 70×10^{-5} equivalents of dye. Acid titration data lie between 75×10^{-5} and 90×10^{-5} moles per gram. The calculated value is 98×10^{-5} equivalents. The lack of correlation between the calculated value and that found by treating the protein with dye may, in part, be due to inaccuracies in the analytical values for the content of amino acids.

In the calculations it is generally assumed that the dissociation constants which have been obtained on amino acids can be applied to the free acidic or basic groups of the protein molecule. This assumption may not be strictly correct since it neglects the steric effects of adjacent groups. The magnitude of these effects has not yet been ascertained. The assumption probably represents a close approximation of the facts.

The question has been raised as to whether the union which takes place between proteins and dyes is chemical in nature or a phenomenon which has been termed adsorption (16, 19). Gortner (19) believes that between certain pH limits the reaction between dve anion and positively charged protein is not necessarily a reaction between primary valences but may be regarded as an electrokinetic phenomenon. The data which have been cited on the combining capacities of proteins for acidic and basic dyes are interpreted by Chapman, Greenberg and Schmidt as indicating that the reaction is a chemical one and takes place in stoichiometric proportions. The data of Hewitt (20), Stearn (21), and Rakusin (22) point in the same direction. The technique used by Stearn (21) deserves some comment. It consists in determining the conductivities of a solution of dye to which varying amounts of protein are added and vice versa. A break in the titration curve indicates the end-point. In the case of gelatin, which was titrated with methyl violet, 71×10^{-5} equivalents of dye were bound per gram of protein. This is practically the same value as that found by Rawlins and Schmidt (23).

In order to obtain additional information on the mode of combination which takes place between proteins and dyes, Rawlins and Schmidt (23) determined the amounts of various dves which are taken up by gelatin granules. The technique consisted in weighing out two samples of gelatin granules and adding to each the same amount of distilled water. One gelatin sample was dissolved with the aid of heat, and the other was permitted to remain as granules. A definite amount of 0.1 N HCl was added to each. This was followed by the addition of varying amounts of dye. After standing a sufficient length of time, the granules were filtered off (or, in the case of the dissolved gelatin, the precipitate was filtered), and the amount of dye which was present in the filtrate was estimated. A typical curve is shown in Fig. 7. It is evident that the amount of dye which the gelatin granules absorb depends on the time during which the granules are in contact with the dye, the acidity of the solution, and the concentration of dye. However, the maximum amounts of dve which were taken up by the gelatin

granules at the maximum periods of time employed were the same as those which were found to combine with gelatin when the latter was in solution. The S-shaped form of the dotted curve in Fig. 7 is indicative of the protective colloidal effect of the protein in preventing precipitation of the protein-dye compound.

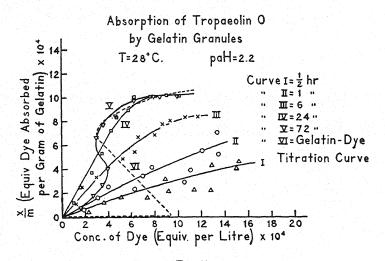


Fig. 7
(Rawlins, L. M. C., and Schmidt, C. L. A., J. Biol. Chem., 88, 271 (1930).)

One of the arguments which has been used in favor of adsorption is that combination between protein and dye takes place in accord with the adsorption isotherm,

$$\frac{x}{m} = KC^{1/n}$$

where $x = \mathrm{dye}$ absorbed, $m = \mathrm{mass}$ of protein, $C = \mathrm{equilibrium}$ concentration, and K and 1/n are constants. Robertson (24) has shown however, that, under certain conditions, this formula can be derived from the mass law and it is, therefore, not a true criterion for adsorption. The data which are given in Fig. 7 have been plotted logarithmically in Figs. 8 and 9. The curves which approach most nearly to a straight line are those which represent experiments in which gelatin granules were permitted to remain in contact with the dye solution for relatively short periods of time. However, the curves which represent maximum periods of time deviate considerably from a straight line. It is apparent that the combination

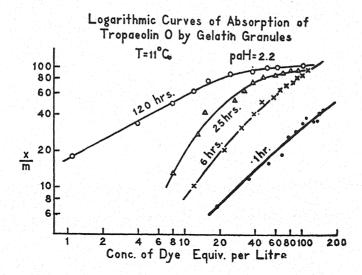


Fig. 8 (Rawlins, L. M. C., and Schmidt, C. L. A., J. Biol. Chem., 88, 271 (1930).)

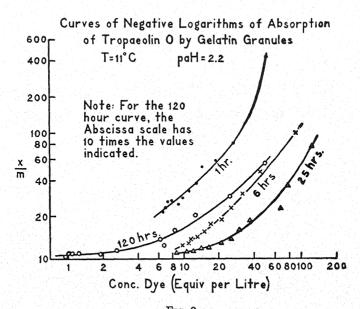


Fig. 9 (Rawlins, L. M. C., and Schmidt, C. L. A., J. Biol. Chem., 88, 271 (1930).)

between gelatin granules and dye does not follow the adsorption isotherm.

(3) Application of the Phase Rule

The acid- and base-combining capacities of amino acids and proteins may be determined by treating them in the solid state with gaseous acids (HCl, H₂S, CO₂) or base (NH₃). This procedure was suggested by Bancroft and his co-workers (25). The procedure consists in treating the solid compound with the gas and determining the dissociation pressure. On addition of gaseous acid or base to solid amino acid or protein, the pressure of the gas will

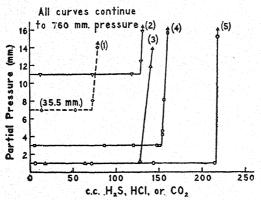


Fig. 10. Gasometric titration curves showing combination of the basic amino acids with hydrogen chloride, carbon dioxide, and hydrogen sulfide. Curve 1 shows 1 gm. of histidine with hydrogen sulfide; Curve 2, 1 gm. of arginine with hydrogen sulfide; Curve 3, 1 gm. of arginine with carbon dioxide; Curve 4, 1 gm. of lysine with hydrogen sulfide; Curve 5, 1 gm. of histidine with hydrogen chloride. The compound which histidine forms with hydrogen sulfide has a dissociation pressure of 35.55 mm. The absolute values on the horizontal portion of Curves 3 and 5 on account of the low pressures are not very accurate.

(Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem., 105, 301 (1934).)

remain constant as long as combination between gas and solid takes place. When combination is complete, there will be a decided increase in pressure upon further addition of gas. Since not more than a trace of water is present in the system, it is not a factor which influences the equilibrium. In fact, most proteins so used have probably not been moisture-free.

The application of the phase rule to this system is made evident from the following discussion. The phase rule is given by

$$F = C - P + 2 \tag{9}$$

In this system there are present two components, gas and amino acid or protein; and three phases, solid amino acid or protein, the

compound which is formed between the amino acid or protein and the gas, and gas. There is then one degree of freedom. Since the gaseous titration is carried out at constant temperature, that degree of freedom is removed. On addition of varying amounts of gas, the pressure must remain constant. This will continue until

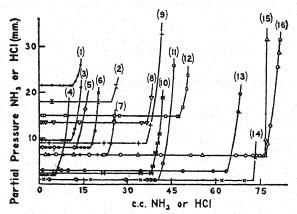


Fig. 11. Gasometric titration curves showing combination of certain proteins with ammonia or hydrogen chloride. Curve 1 shows 1 gm. of globin with ammonia; Curve 2, 1 gm. of edestin with ammonia; Curve 3, 1 gm. of hemoglobin with ammonia; Curve 4, 1 gm. of zein with hydrogen chloride; Curve 5, 1 gm. of zein with ammonia; Curve 6, 1 gm. of gelatin with ammonia; Curve 7, 0.5 gm. of hemoglobin, with ammonia; Curve 8, 1 gm. of crystalline egg albumin with ammonia; Curve 9, 1 gm; of edestin with hydrogen chloride; Curve 10, 1 gm. of crystalline egg albumin with hydrogen chloride; Curve 11, 1 gm. of globin with hydrogen chloride; Curve 12, 1 gm. of casein with ammonia; Curve 13, 1 gm. of casein with hydrogen chloride; Curve 14, 0.5 gm. of salmin with hydrogen chloride; Curve 15, 1 gm. of gelatin with hydrogen chloride; Curve 16, 1 gm. of gelatin with hydrogen chloride (reverse titration with the same sample which was used for Curve 15). This reverse titration was made by withdrawing known amounts of hydrochloric acid gas from the system, and plotting the changes in pressure against the number of cubic centimeters of the gas withdrawn. Curves 15 and 16 meet at a pressure of 200 mm. A reverse titration serves as a check on the forward titration.

(Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem. 105, 301 (1934).)

all of the amino acid or protein has combined with the gaseous acid or base. At this point only two phases, gas and amino acid or protein compound, are present. We have then two degrees of freedom and since one of them, temperature, is fixed, the pressure in the system will change. If chemical combination between gas and solid does not take place, then the system consists of only two phases and the system is bivariant. Since the temperature is fixed the pressure will vary continuously.

The phase rule may also be applied to a system in which the acid or base is dissolved in some appropriate solvent, such as alcohol, which is chemically inert to the system, and which does not dissolve the product which is to be titrated or the compound formed. Varying amounts of acidic or basic solution are added to constant amounts of solid and the mixture is made up to constant volume by addition of solvent. When equilibrium is reached, a

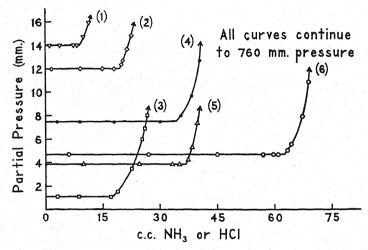


Fig. 12. Gasometric titration curves showing combination of certain proteins with ammonia or hydrogen chloride. Curve 1 shows 1 gm. of degummed silk fibroin with hydrogen chloride; Curve 2, 1 gm. of degummed silk fibroin with ammonia; Curve 3, 1 gm. of deaminized gelatin with ammonia; Curve 4, 0.5 gm. of deaminized gelatin with hydrogen chloride; Curve 5, 1 gm. of dephosphorized casein with ammonia; Curve 6, 1 gm. of dephosphorized casein with hydrogen chloride.

(Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem., 105, 301 (1934).)

definite amount of the supernatant fluid is withdrawn and titrated. If a compound is forming and, until it is complete, there are present in the system two solid and a liquid phase and two components. Since the experiment is carried out at constant temperature, the system is invariant, indicating that the liquid will contain no acid or base until compound formation is complete. After that the supernatant liquid will contain acid or base.

Use of the method of gaseous titration has been made by Czarnetzky and Schmidt (26). Their data are graphically represented in Figs. 10 to 12. The dissociation pressures at various temperatures, when the amino acids were treated, respectively, with hydrogen chloride or ammonia, are given in Tables V to VII. With

the following exceptions, the number of equivalents of HCl that combine with the given compounds was determined by the number of equivalents of aliphatic amino groups in the molecule. The exceptions are arginine, histidine, and tryptophane, which combine

Table V¹

Dissociation Constants and Thermodynamic Data of Amino Acids at 25°

Amino acid-NH₃(s) ⇒ amino acid (s) + NH₃(g)

Amino acid	pk′ _a *	$pK_a{}^s$	$\Delta H p \mathbf{K_{a}}^{s}$	$\Delta F \mathrm{pK}_{m{a}^8}$	$\Delta S_{ m pK}_a{}^s$	Dissociation pressures at			
						2.5°	25°	40°	
						mm.	mm.	mm.	
Alanine	9.69	1.876	6680	2560	13.89	4.30	10.05	17.40	
α-Aminobutyric acid	9.60	1.873	6770	2560	14.12	4.25	10.15	17.65	
α-Aminocaprylic acid		1.722	7000	2350	15.60	6.30	14.45	25.40	
Aspartic acid	3.70	2.449	2840	3340	-1.68	1.75	2.70	3.40	
Aspartic acid	9.70	1.865	6690	2530	14.49	4.20	10.55	18.20	
Cystine	7.48	1.980	7280	2700	15.36	3.10	7.95	14.35	
Diiodotyrosine	6.48	2.039	6820	2780	13.55	2.40	6.95	12.05	
Clustomic soid	4.25	2.602	3630	3550	0.27	1.20	1.90	2.55	
Glutamic acid	9.60	1.860	6780	2530	14.25	4.15	10.45	18.10	
Glycine	9.60	No re	eaction						
Hydroxyproline	9.73	2.152	6140	2940	10.73	2.30	5.35	8.85	
Isoleucine	9.68	1.877	6800	2560	14.22	3.95	10.10	17.55	
Isoserine	9.27	1.907	7190	2600	15.39	3.75	9.40	16.95	
Leucine	9.60	1.900	7050	2590	14.96	3.85	9.55	16.95	
Methionine	9.21	1.893	7020	2580	14.89	3.80	9.70	17.15	
Norleucine	9.76	1.867	7140	2550	15.39	4.00	10.30	18.40	
Norvaline	9.72	1.870	7170	2550	15.50	3.90	10.25	18.35	
Phenylalanine	9.23	1.910	7220	2600	15.50	3.70	9.35	16.80	
Proline		2.046	5120	2790	7.82	0.95	6.85	13.00	
Serine	9.15	1.903	7140	2600	15.23	3.80	9.50	16.95	
Tryptophane	9.39	1.854	6770	2530	14.22	3.75	10.65	18.40	
Tyrosine	9.18	1.830	7110	2500	15.46	3.30	11.30	20.00	
Valine	9.62	1.870	7170	2550	15.10	3.90	10.30	18.35	

^{*} The values for pk'_a were taken from the table published by Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 90, 165 (1931).

(Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem., 105, 301 (1934).)

with 2, 3, and 2 equivalents, respectively. These three amino acids combine with only one equivalent of carbon dioxide or of hydrogen sulfide. The basic amino acids combine with one mole of carbon dioxide and one mole of water, indicating that the respective bicarbonates are formed. With the exception of arginine,

¹ In Tables V, VI and VII $s=\mathrm{solid},\,g=\mathrm{gas}.\,\Delta F-\Delta H=-T\Delta S$ (see Chapter XV for the significance of these terms.)

lysine, and histidine, the number of equivalents with which a compound combines corresponds to the number of carboxyl groups in the molecule. Although the amide nitrogen of glycine anhydride, of asparagine, and of the simple peptides does not react with hy-

Table VI

Dissociation Constants and Thermodynamic Data of Amino Acids at 25°

Amino acid-HCl(s)

Amino acid (s) + HCl(g).

Amino acid	pk′ _b *	$\mathrm{pK}_b{}^s$	ΔH p K_b s	$_{\Delta F} \mathrm{pK}_{b^8}$	ΔS p ${ m K}_b{}^s$		ssociat essures	
						2.5°	25°	40°
						mm.	mm.	mm.
Alanine	11.66	2.456	2900	3350	-1.51		2.65	3.35
α-Aminobutyric acid	11.45	2.466	2930	3370	-1.48		2.60	3.30
α-Aminocaprylic acid		2.418	2840	3300	-1.54		2.90	3.65
Aspartic acid	12.00	2.017	6540	2750	12.71	2.65	7.30	12.40
Cystine	12.33	2.611	3720	3560	+0.54		1.85	2.50
Diiodotyrosine†	11.88							
Glutamic acid	11.85	1.957	7880	2670	17.47	2.80	8.40	15.90
Glycine	11.66	2.500	2760	3410	-2.18		2.40	3.00
Hydroxyproline	12.08	2.580	2270	3520	-4.19		2.00	2.40
Isoleucine	11.64	2.440	2900	3330	-1.44	2.10	2.75	3.45
Isoserine	11.22	2.212	2840	3020	-0.60		4.65	5.85
Leucine	11.64	2.450	2840	3340	-1.68	2.05	2.70	3.40
Methionine	11.72	2.450	2840	3340	-1.68		2.70	3.40
Norleucine	11.61	2.440	2820	3330	-1.71	2.05	2.75	3.45
Norvaline	11.64	2.446	2960	3340	-1.27		2.60	3.30
Phenylalanine	11.42	2.448	2840	3340	-1.68		2.70	3.40
Proline	12.00	2.556	3550	3490	+0.20		2.10	2.80
Serine	11.79	2.236	2300	3050	-2.52		4.40	5.30
Tryptophane ‡	11.66	2.560	6450	3490	9.92	100	2.10	2.80
Tyrosine	11.76	2.473	2990	3380	-1.31		2.55	3.25
Valine	11.68	2.446	2930	3340	-1.38	41 st.	2.60	3.30

^{*} The values for pk'a were taken from the table published by Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 90, 165 (1931).

drogen chloride, it is interesting to note that the peptide linkage of alanylproline does so. The data which are given in Tables V to VII indicate that the amino acids, when in the dry state, exist almost wholly as zwitterions.

From the foregoing data it might be expected that the following groups, when present in the protein molecule, will react with hydrogen chloride: the two nitrogen-containing groups in the imida-

[†] Splits off iodine when treated with HCl.

[‡] The second dissociation constant is identical with the first.

⁽Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem., 105, 301 (1934).)

zole ring of histidine, the ϵ -group of lysine, the guanidine ring of arginine, the imino group of tryptophane, and the imino groups of proline and hydroxyproline (since they are present in the protein molecule as -CON- groups). Similarly, it would be expected that gaseous ammonia would combine with the free carboxyl groups of the dicarboxylic amino acids (computed on the basis that each dicarboxylic amino acid yields a free carboxyl group less those which are present as acid amide groups), the carboxyl group of any amino acid except glycine, and the free phosphoric acid groups.

Table VII

Titration of Basic Amino Acids with Hydrogen Sulfide Gas

Amino acid- $H_2S(s) \rightleftharpoons amino acid (s) + H_2S(g)$.

Amino acid	pk′ _a *	pk'_b	\mathbf{K}_{I}	pK_{b^8}	$\Delta H \mathrm{pK}_{b^S}$	ΔF ր ${ m K}_b$ ፥	$\Delta S \mathrm{p} \mathrm{K}_{b^{8}}$		ssociatio pressures	
<u> </u>								2.5°	25°	40°
								mm.	mm.	mm.
Arginine.	9.70	11.83 5.70		1.842	2270	2510	-0.80	7.85	11.00	13.35
Histidine	9.41	$12.52 \\ 7.92$	1.	1.332	1140	1820	-2.28	20.25	35.55	39.05
Lysine	10.52	12.00 4.70		2.405	1000	3280	-7.31	2.65	3.00	3.25

^{*}The values for pk'a and pk'b were taken from the table published by Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 90, 165 (1931). (Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem., 105, 301 (1934).

It would also be expected that titration of proteins with hydrogen chloride should yield values which are greater than those which have been obtained when proteins are titrated in aqueous solution with hydrochloric acid. Treatment with hydrogen chloride was found to result in a small amount of hydrolysis of the protein which increases its acid-combining capacity. The hydroxy group in the phenyl ring of tyrosine does not react with gaseous ammonia. If the protein contains tyrosine it would be expected that titration of aqueous protein solutions with alkali would yield values which are somewhat greater than those which treatment with gaseous ammonia will yield. However, it should be remembered that titration of a protein in aqueous solution is not so complete as in the case of the gaseous titration. A comparison of the values for the acid- and base-combining power of a number of proteins which have been reported is given in Table VIII. According to early

Table VIII

Acid- and Base-combining Power of Certain Proteins

Protein	Acid bound	Base bound	${f Method}$
	moles × 105	moles×105	
	per gm.	per gm.	
Casein*		160	Potentiometric.
Cusom	100	210	Dye titration.
	100	183	Potentiometric.
	60, 76, 90,	155, 136, 140	Potentiometric.
	59, 72	100, 100, 110	Potentiometric, recalculated val-
	03, 12		ues of Hitchcock and Loeb.
	95	180	Calculated.†
	233	100	Gas titration.
	284‡	212	Gas titration present work.
	197	150	Calculated.
Gelatin	103	150	Dye titration.
Gelaulii	103	60	Potentiometric.
	92, 89, 86	56, 57	Potentiometric, recalculated val-
	92, 39, 30	30, 37	ues of Loeb and Hitchcock.
	89	75	Conductometric titration.
	89	10	Potentiometric.
	92	70	Potentiometric.
	107	41	Calculated.†
	302	71	Gas titration.
	342‡	78	
	317	41	Gas titration, present work.
Deaminized gelatin	59	41	Calculated §
Deaminized geraum	45		Dye titration.
	67	41	Potentiometric.
	305	80	Calculated.†
	276	41	Gas titration, present work.
Edestin	157	70	Calculated.§
12desum	107	75	Dye titration. Potentiometric.
		90	
	127	90	Potentiometric.
	121		Potentiometric, recalculated val-
	133	98	ues of Kodama and of Hitchcock
	164	109	Calculated.†
	210	73	Gas titration, present work. Calculated.
Egg albumin	80	80	
Dag aroumm			Potentiometric, recalculated val- ues of Hitchcock and of Berggren
	110	134	Potentiometric.
	65	81	Calculated.†
	170	162	Gas titration, present work.
	113	58	Calculated. §,
Zein	0	28-31	Potentiometric.
	16	61	Calculated.†
	24	56	Gas titration, present work.
	100	28	Calculated.§
Globin (ox)¶	22	23	Potentiometric.

TABLE VIII-Continued

Protein	Acid bound	Base bound	${f Method}$
	moles × 10 ⁵	moles × 10 ⁵	
	per gm.	per gm .	
Globin (ox) ¶ $(Cont.)$	131	71	Calculated.†
	181	51	Gas titration, present work.
	243	45	Calculated.§
Hemoglobin (ox)¶,**	146		Calculated.†
	69	69	Potentiometric.
	126	68	Calculated.†
	210	48	Gas titration, present work.
	234	43	Calculated.§
Salmin	496		Analysis.
	502		Calculated.†
	648		Gas titration, present work.
	597		Calculated.§
Silk fibroin¶	19	55	Calculated.†
	40	86	Gas titration, present work.
	33		Calculated.§
Dephosphorized ca-			
sein¶	280‡	170	Gas titration, present work.

* The phosphoric acid has not been considered in the calculated values of this and other phosphorus-containing proteins.

† The calculated values for the acid-combining capacity are based on the assumption that arginine, histidine, and lysine each contribute one basic group. The calculated amount of base with which the protein should combine is based on the assumption that glutamic acid, hydroxyglutamic acid, aspartic acid, and tyrosine each contribute one acid group less the amount which is combined as acid amides (based on the amount of ammonia liberated on hydrolysis). The values are those which might be expected when the protein is in aqueous solution.

‡ The values given in parentheses have been corrected for increase of amino nitrogen due to hydrolysis. When no such figures are given, no apparent hydrolysis took place.

§ The calculated values for the acid-combining capacity are based on the assumption that arginine, lysine, proline, hydroxyproline, and tryptophane each contribute one and histidine contributes two basic groups. The calculated values for the base-combining capacity are based on the assumption that aspartic acid, glutamic acid, and hydroxyglutamic acid each contribute one acid group less the amount which is combined as acid amides (based on the amount of ammonia liberated on hydrolysis). The values are those which might be expected when the solid protein is treated with hydrogen chloride or ammonia gas.

 \parallel On the basis of the analyses published by Calvery, the calculated combining power of egg albumin for acids is 146×10^{-5} and for bases 67×10^{-5} moles per gm. of protein.

¶ Since the analytical values for the content of amino acids are incomplete or

not very reliable, the calculated values are necessarily not very reliable.

** The calculated values for hemoglobin are based on the content of globin which constitutes about 96 per cent of the hemoglobin molecule. (A recent compilation of acid- and base-combining capacities of proteins has been made by Cohn, E. J., Ergeb. Physiol., 33, 781 (1931). See also Green, A. A., J. Amer. Chem., Soc., 60, 1108 (1928).

(Czarnetzky, E. J., and Schmidt, C. L. A., J. Biol. Chem., 105, 301 (1934).)

potentiometric titration data, zein was reported as not combining with hydrochloric acid in an aqueous medium; nevertheless, gaseous titration shows that it combines with 24×10^{-5} moles of hydrogen chloride per gram. Cohn and his co-workers (27) have more recently shown that, in alcoholic solution, zein combines with 17.8 to 21.3×10^{-5} equivalents of hydrochloric acid. The dissociation constants of zein are: pK₁′ = 5.4, pK₂′ = 6.9.

Discussion of the titration curves of aqueous solutions of amino acids with acids and bases is given in Chapter XI.

2. COMBINATION OF AMINO ACIDS AND PROTEINS WITH HEAVY METALS

(1) Iron

In Chapter XII evidence was presented to show that proteins, when combined in solution with strong acids, ionize to yield hydrogen and protein ions, and, when combined with the alkali hydroxides, yield alkali and protein ions. The alkaline earth hydroxides, due to step dissociation, yield complex protein-alkaline earth ions. It is wholly probable that all of the heavy metals form complexes with proteins so that the activity of the heavy metal is small. Certain phases of this subject have been discussed in Chapter XII. The term, "metallic complex," refers to that class of chemical compounds which are formed (a) between a positively charged metallic ion and either a negatively charged inorganic or organic ion to produce (b) a compound which is soluble in aqueous solution in which (c) the activity of the metallic ion is greatly reduced and (d) in which the charge carried by the compound so formed may be equal to, but, in most cases, is different from that of the constituent metallic element either in sign or in magnitude.

In discussing this subject, the work of Smythe and Schmidt (28) may be cited for illustrative purposes. They studied the mode of combination of amino acids and proteins with ferric iron. The method employed in determining the ferric iron concentration consisted in adding the substances to be tested to a known solution of ferric iron. To this a standard amount of ammonium thiocyanate was added, and the reaction was adjusted to a definite pH. The color of this solution was compared with that of a standard ferric thiocyanate solution. In every case the standard contained the same amount of thiocyanate and had the same pH as the solution compared with it. If the substance forms a compound with iron (e.g., complex ion), which is less dissociated than ferric thiocyanate,

the color of the solution will be less intense than that of the standard. If the ferric compound is dissociated to a greater extent than ferric thiocyanate, no appreciable effect on the color will be produced. At a given pH an equilibrium exists in these solutions between iron, thiocyanate, and the substance under test. If any

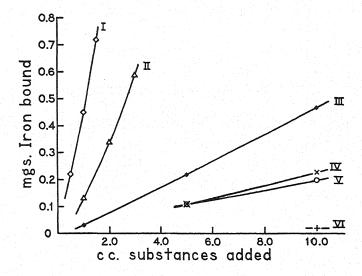


Fig. 13. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.005 M oxalic acid; Curve II, 0.01 M malonic acid; Curve III, 0.01 M maleic acid; Curve IV, 0.01 M succinic acid; Curve V, 0.01 M glutaric acid; Curve VI, 0.01 M adipic acid.

(Smythe, C. V., and Schmidt, C. L. A., J. Biol. Chem., 88, 241 (1930).)

two of these and the pH are kept constant, the effect of varying the concentration of the other can be determined. In most cases the pH was adjusted to 2.5. This is about the upper limit at which this method can be used conveniently and accurately, due to the marked insolubility of ferric hydroxide and the susceptibility of the color to very slight changes in pH in more alkaline solutions. Certain of the data are represented in Figs. 13 to 16.

Migration experiments were also carried out. If the iron in a solution, containing (in addition to the iron) only the substance under test, is present in the anodic condition as shown by migration experiments, it must be combined in the form of a complex ion. Due to the high acidity of the solution, quantitative transference experiments cannot be carried out. The migration data are given in Table IX.

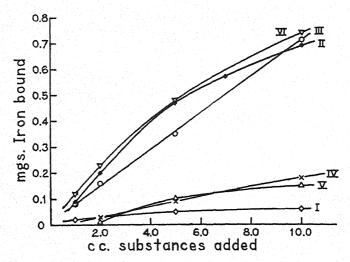


Fig. 14. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.01 M serine; Curve II, 0.01 M asparatic acid; Curve III, 0.01 M glutamic acid; Curve IV, 0.01 M asparagine; Curve V, 0.01 M creatine; Curve VI, 0.01 M β -hydroxyglutamic acid.

(Smythe, C. V., and Schmidt, C. L. A., J. Biol. Chem., 88, 241 (1930).)

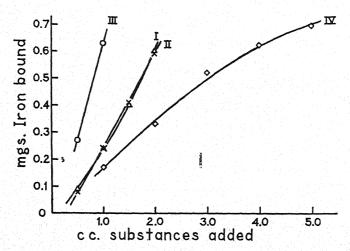


Fig. 15. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 0.94 per cent casein; Curve II, 1.00 per cent dephosphorized casein; Curve III, 1.00 per cent dephosphorized casein plus the phosphoric acid split off from it; Curve IV, 1.52 per cent gelatin.

(Smythe, C. V., and Schmidt, C. L. A., J. Biol. Chem., 88, 241 (1930).)

The results indicate that the following substances form complexes with ferric iron: compounds which possess an hydroxy group in the α -position with respect to the carboxyl group (when the hydroxy group is in the β -position the influence is less), dicarboxylic

acids (introduction of CH₂ groups between the carboxyl groups lessens the effect), inorganic acids, such as orthophosphoric, which have a structure similar to that of hydroxy organic acids, and proteins. The following show no evidence of forming complexes with ferric iron: substances which possess hydroxyl groups, but which either are not acids or are very weak acids, amino acids other than the dicarboxylic amino acids, and the simple peptides,

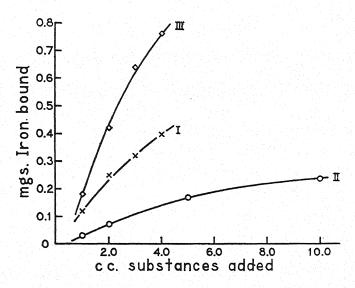


Fig. 16. Total iron present 1.12 mg.; total volume 25 cc.; Curve I, 1 per cent protamin sulfate; Curve II, 1 per cent protamin; Curve III, 0.0025 M yeast nucleic acid.

(Smythe, C. V., and Schmidt, C. L. A., J. Biol. Chem., 88, 241 (1930).)

such as glycylglycine. On the basis of these data a comparison of the expected amount with the actual amount of iron which was found to be combined with casein and gelatin can be made. On the assumption that the groups which react in the protein molecule exert the same effect as the free amino acids, 1 cc. of a 1 per cent casein solution should bind 0.21 mg. of iron. Experimentally, the value found was 0.24 mg. Two cc. of a 1.52 per cent gelatin solution should combine with 0.11 mg. of iron; the experimental value was 0.33 mg. The correlation is an approximate one.

An explanation for the behavior of iron in forming complexes is found in the following. In order that a ferric compound may be undissociated in the presence of an excess of thiocyanate, it is necessary that some force, in addition to the usual valence bond, act on the iron atom to hold it in position. This force may be

Table IX

Migration of Iron in Solutions Containing Various Substances

		Origina	al soluti	ion	A	node po	rtion	M	iddle p	ortion	Ca	thode p	portion
Substance added	pН	Molality of substance being tested	Iron per cc.	Total iron	рН	Iron per cc.	Total iron	Нq	Iron per cc.	Total iron	pН	Iron per cc.	Total iron
Acetic acid.	2.5	1.0	mg. 1.97	mg. 246.00	2.4	mg. 1.77	mg. 72.50	2.4	mg. 1.97	mg. 80.77	2.8	mg. 2.24	mg. 96.20
Lactic acid.	2.0 7.1	0.1 0.1	1.04 0.54	130.00 62.91	1.8 4.0		33.97 21.09	2.0 6.0	1.04 0.50	43.68 21.25	2.5 10.7		51.60 18.50
Mandelic acid	1.9 7.0	0.1 0.1	0.49 0.24	60.27 27.24	1.7 3.7		19.32 9.00	2.3 9.0		16.40 8.51	2.0 11.4		22.55 8.14
Oxalic acid.	2.1 6.5	0.01 0.1	0.49 0.10	60.76 11.20	$\frac{1.6}{2.7}$		26.15 4.32	1.9 9.0		21.42 3.20	4.5		15.80 3.24
Malonic acid	1.5	0.1	1.45	175.45	1.4	1.60	64.00	1.6	1.51	60.40	1.8	:	48.38
Citric acid.	2.1 7.0	0.01 0.1	0.31 0.51	37.98 57.63	1.9 4.0		19.32 19.80	3.1 9.0	$0.33 \\ 0.49$	13.04 20.09	4.9 9.6		6.56 15.84
Tartaric acid	2.1 7.0 10.0	0.01 0.1 0.1	0.25 0.61 0.58	30.25 68.32 63.51	$2.0 \\ 2.5 \\ 3.5$	0.76	10.53 27.36 23.10	2.0 7.0 10.3	0.55	11.48 22.00 21.33	2.8 9.5 10.4	0.46	9.02 16.56 17.85
Saccharic acid	10.7	0.1	0.20	21.20	4.1	0.22	7.26	10.7	0.19	7.60	10.8	0.17	5.61
Orthophos- phoric acid	1.9	0.1	0.25	32.25	1.9	0.18	7.56	1.9	0.24	9.84	2.0	0.28	12.88
Pyrophos- phoric acid	1.4	0.1	0.14	17.36	1.0	0.20	8.40	1.4	0.14	5.74	1.9	0.09	3.69
Glycerophos- phoric acid	1.5	0.1	1.03 3.50*		1.5	0.94 3.90*	39.48 163.80*	1.4	0.97 3.50*	39.29 141.75*	1.7	1.13 3.30*	47.46 138.60*
	3.7 6.5	0.1	0.56 0.75	70.00 69.75	3.4 5.4		24.49 26.40	5.0 6.7	0.56 0.72	23.52 19.08	6.3 10.5		21.17 23.12
Glutamic acid	2.7 4.0 5.0	0.075 0.075	0.15 0.15 +	18.30 18.68	2.4 2.6 3.0		2.52 4.25	3.1 4.2 5.2	0.12 0.16 +	4.68 6.40	3.4 7.5 7.8		9.84 7.98
Aspartic acid	7.0 7.4		+ +			++ ++.			+ +			=	
Casein	2.4 7.2	1% 1%	0.26 0.17	28.73 15.73	2.3 5.0	0.20 0.20	7.00 6.60	2.6 8.3	0.24 0.18	9.96 5.04	3.8 10.0		10.20 4.41
Gelatin	2.2 7.0	1 % 1 %	0.18 0.19	20.34 20.90	1.7 3.8	0.16 0.20	5.76 6.80	4.1	0.20 0.20	8.20 8.40	2.5 7.5	0.22 0.18	7.94 6.12

^{*} These figures refer to estimations of phosphorus. (Smythe, C. V., and Schmidt, C. L. A., J. Biol. Chem., 88, 241 (1930).)

termed secondary valence, coordinative valence, or the attraction of charges. The difference in behavior between propionic and lactic acid must be ascribed to the hydroxy group. Now oxygen can become tetravalent and it may do so here. A simpler explanation and one which is more in accord with modern electronic concepts of valence is the following. It is possible to compute the residual charge on the oxygen atom in the manner proposed by Latimer and Porter (29). The electronic arrangement of this atom is as follows:

Н :ö: —ён

The oxygen atom has a kernel charge of 6+. It has 4 electrons free, 2 shared with hydrogen, and 2 shared with carbon. The hydrogen ion is considered sufficiently close to the center of the negative charges between it and the oxygen to just neutralize 1 electron. The 2 electrons shared with carbon will be shared not equally, but in the ratio of the kernel charges of oxygen and carbon. These are 6+ and 4+, respectively, so that the oxygen will have 0.6 of these 2 electrons. Summing up the positive and negative charges: $6-4-(2\times 1/2)-(2\times 0.6)=-0.2$. According to this calculation the oxygen atom actually possesses a residual negative charge. This will exert an attraction on the positive iron. The compound formed may be represented as

 $CH_3 \cdot \overset{H}{C} \cdot \overset{O}{C}$ $HO ---- Fe(X)_n$

where X represents any negative group. This attraction, in addition to the usual bond, may be sufficient to prevent the dissociation of iron. If the thiocyanate ion is considered as having the structure $(SC \equiv N)^-$, then the ferric thiocyanate itself may be a compound of the above type, or

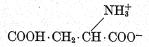
$$SC \equiv N$$
 (X) . Fe

If the hydroxyl group is moved back to the β -carbon atom, its residual charge remains the same, but the distance between it and the iron has increased so that it produces a smaller effect. The same considerations will explain the effect of the dicarboxylic acids. The effect of the monocarboxylic amino acids falls in line with this reasoning. If they exist in solution in the classical form, the nitrogen possesses a residual charge of -0.11. They should then produce an effect. If they exist in the zwitterion form, the residual charge on the nitrogen is +0.89. They should then show no effect. In acid solutions practically all of the nitrogen is in the form of NH₃, and hence the amino acid should not form a complex with iron. This is in accordance with the experimental findings.

The dicarboxylic amino acids produce an effect which is much greater than that of the unsubstituted acids. This is not indicative that the amino group participates in the union with iron. Two factors are necessary for the formation of iron complexes. One of these is a valence bond, the other is an additional attraction from some other part of the molecule. Then, if other conditions are equal, the amount of iron compound which is formed is a function of the concentration of the group which unites with iron by means of the bond. In the case of succinic, maleic, and aspartic acids, one carboxyl group furnishes the bond, the other furnishes the additional attraction. Solutions of the same molality, at the same

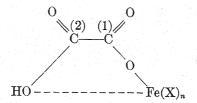
pH, differ only in the concentration of the — group which

furnishes the bond. Since maleic acid, when brought to the same pH, is a stronger acid than succinic, it will be more ionized and hence offer a greater concentration of the group which unites with iron. If aspartic acid exists as the zwitterion, it offers a still greater concentration of this group, since at pH 2.5 it exists largely as



At this pH serine, however, will exist largely as

In the case of the dicarboxylic acids the iron-containing compound may be represented as



The iron in this molecule is very slightly dissociated. If carboxyl group (2) ionizes, then the iron is present in the anodic form. If the pH is such as to repress entirely the ionization of group (2), then the only iron which will move with the current is that which dissociates from the above compound and this will be positively charged. By varying the pH of the solution the direction of migration of the iron may be changed. Phosphoric acid is present in proteins in the form of an ester and is probably combined with the hydroxyl group of serine or other hydroxy acids. The influence of this type of acid on iron may be illustrated by glycerophosphoric acid. At pH 1.5 the iron is cathodic. This means that the only iron which migrates with the current is that which is free according to the following dissociation:

RO O RO O
$$+F_{e}(X)$$
,

HO $----F_{e}(X)$

Or, if we consider that X is different from the glycerophosphoric radical, we may have

RO O RO O
$$P \Rightarrow P + X^{-}$$

$$O \rightarrow O$$

$$HO -----Fe(X)_{n} \quad HO -----Fe(X)_{n-1}$$

If the pH is increased to 3.7, the following dissociation is able to take place:

and the iron becomes anodic. The reason that iron in pyrophosphoric acid is anodic at such a low acidity is that the second hydroxyl group of this acid is strongly acidic. It has a K_a value of 1.1×10^{-2} . Anodic iron was not obtained in glutamic acid solutions due to the fact that the iron is precipitated as the hydroxide before the second carboxyl group can be made to ionize.

(2) Manganese

The above principles can also be applied to the study of the complexes which divalent manganese forms with amino acids and proteins. The criterion used to determine complex formation by Main and Schmidt (30) consisted in determining colorimetrically the relative concentration of manganous ions in the presence of the substance being tested for its ability to form complex ions. For this purpose the color which is produced in a chloroform solution of isonitrosoacetophenone, when shaken with an aqueous phase containing manganous ions and the test substance, was utilized. The aqueous solution was adjusted to pH 9.25 by means of a borate buffer.

The validity of the distribution experiments depends upon sound theoretical considerations. In the aqueous phase, A, (containing Mn^{++} , HBO_2 , Na^+ , BO_2^- , X^- , where X^- represents the substance under test), the following equilibria exist:

(a)
$$HBO_2 + OH^- \rightleftharpoons H_2O + BO_2^-$$

(b)
$$Mn^{++}+X^- \rightleftharpoons Mn X \text{ (complex)}$$

In the chloroform phase, C, $(Mn^{++}, Mn(Ox)_2, Ox^-, where Ox$ represents isonitrosoacetophenone), the significant equilibrium is

(c)
$$Mn^{++}+2 Ox \rightarrow Mn(Ox)_2$$
 (colored complex)

Between the two phases there exists the significant equilibrium in the case of manganous ions:

At equilibrium the fugacity of Mn⁺⁺ in phase A is equal to the fugacity of Mn⁺⁺ in C. As the concentration of X⁼ in A is increased, equilibrium (b) is shifted in favor of MnX with the result that equilibrium (d) is shifted towards the left and this, in turn, leads to a like shift in equilibrium (c).

The resulting decrease in the concentration of $Mn(Ox)_2$ is measured colorimetrically. The distribution ratio, R, in the absence of Ox^- and X^- (as in a solution of $MnCl_2$ distributed between water and chloroform),

$$R = \frac{\text{Mn}^{++} \text{ (aqueous phase)}}{\text{Mn}^{++} \text{ (chloroform phase)}}$$

must be very large. The color in the chloroform phase, when Ox and X^- are present, is due to a favorable distribution, not of Mn^{++} , but of the manganous oxime complex

$$R\left[\mathrm{Mn}(\mathrm{Ox})_{2}\right] = \frac{\mathrm{Mn}(\mathrm{Ox})_{2} \text{ (aqueous phase)}}{\mathrm{Mn}(\mathrm{Ox})_{2} \text{ (chloroform phase)}}$$

wherein the distribution ratio favors greatly the chloroform phase. The relation between the concentration of Mn⁺⁺ in the borate buffer and the chloroform solution of oxime can be strictly linear

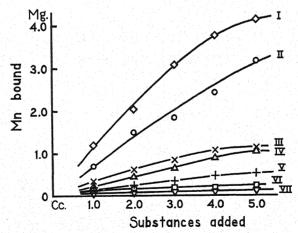


Fig. 17. Total manganese present 5.5 mg.; total volume of aqueous phase 20 cc.; volume of chloroform phase 15 cc.; Curve I, 0.01 M aspartic acid; Curve II, 0.01 M glutamic acid; Curve III, 0.01 M arginine hydrochloride; Curve IV, 0.01 M glycine; Curve V, 0.01 M alanine; (also 0.01 M norleucine); Curve VI, 0.01 M ethylamine hydrochloride; Curve VII, 0.01 M delta-aminonorvaleric acid (also 0.01 M gamma-aminonorvaleric acid).

(Main, R. K., and Schmidt, C. L. A., J. Gen. Physiol. 19, 127 (1935)).

only if (1) the extracting medium is constant and therefore always contains the oxime in large excess, and (2) a small amount only of the total Mn⁺⁺ is removed from the aqueous phase, or (3) the complex in the chloroform layer is very stable and all of the Mn⁺⁺ is extracted. In the latter case, the color intensity should not change above a minimum value with the concentration of oxime which it seems to do. It must therefore be assumed that in the chloroform layer, equilibrium (c) favors largely the dissociated form. To the

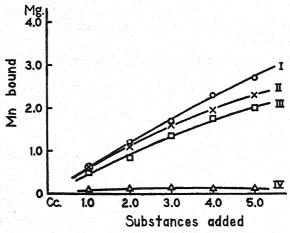


Fig. 18. Total manganese present 5.5 mg.; total volume of aqueous phase 20 cc.; volume of chloroform phase 15 cc.; Curve I, 0.01 M lysine in 0.02 M sodium chloride; Curve II, 0.01 M glycylglycine in 0.01 M sodium chloride; (also 0.01 M leucylglycylglycine); Curve III, 0.01 M leucylglycylglycine (also 0.01 M tyrosine); Curve IV, 0.01 M glycine anhydride.

(Main, R. K., and Schmidt, C. L. A., J. Gen. Physiol., 19, 127 (1935).)

extent that the manganous oxime complex is proportional to the Mn⁺⁺ activity in the aqueous phase, Ox⁻ must be practically constant. The closely linear relation of "bound" Mn to the amount of X⁻ added implies that the complex formed with X⁻ is very stable compared to the water or borate compounds of manganese which, in turn, are very stable compared to the oxime complex. It was shown experimentally that MnX in the aqueous phase is largely undissociated in comparison to Mn(Ox)₂ in the chloroform phase. Moreover, the presence of the borate buffer was shown not to be a disturbing influence in the distribution experiments.

Certain of the data which were obtained in the distribution experiments are shown in Figs. 17 to 19. The results are essentially

Table X
Migration of Manganese in Solutions of Various Substances

	Or	iginal s	olution	Anode portion			liddle ortion	Cathode portion	
Substance added	pН	Molal- ity of sub- stance tested	Manga- nese	pН	Manga- nese	pН	Manga- nese	pН	Manga- nese
Oxalic acid	5.9 9.1	0.1 0.1	per cc. 0.0721 0.185	2.6 4.4	per cc. 0.0687 0.198	5.3 9.7	per cc. 0.0744 0.165	7.7 10.8	per cc. 0.0732 0.188
Malonic acid.	2.0 8.0		0.474 0.900	2.0 5.0	0.404 0.912	2.0 7.6	0.475 0.904	$\frac{2.1}{9.2}$	0.584 0.848
Succinic acid.	7.1	0.1	0.0480	5.6	0.0507	8.1	0.0475	11.4	0.9455
Tartaric acid.	$\frac{2.5}{7.8}$	1	0.0805 0.0780	$\frac{2.2}{4.3}$	0.0710 0.0833	2.5 8.4	0.0802 0.0843	$\frac{2.7}{11.4}$	0.0897 0.0663
Citric acid	7.9	0.05	0.0802	6.0	0.0918	8.7	0.0784	11.5	0.0705
Lactic acid	$\frac{2.8}{6.7}$		0.523 0.0613	$\frac{2.6}{3.9}$	0.376 0.0561	2.8 7.2	0.526 0.0587	3.1 11.4	
Orthophos- phoric acid	8.0	0.10	0.00419	6.7	0.00453	8.1	0.00420	11.3	0.0038
Aspartic acid	4.1 9.0	1.	0.926 0.0717	3.5 3.5	0.648 0.0721	4.1 10.1	0.933 0.0714	5.3 11.2	
Glutamicacid	4.6 9.1	1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 - 1 -	0.900 1.525	4.2 5.2	0.646 1.550	4.6 9.0	0.946 1.526	5.5 10.1	1.103 1.490
Glycerophos- phoric acid	7.6	0.1	0.0313	6.5	0.0332	7.8	0.0306	11.1	0.0296
Casein	2.0 8.5	The second of	0.123 0.0880	$\substack{1.9 \\ 2.2}$	0.109 0.124	2.0 10.5	0.115 0.0915	$\frac{2.2}{11.4}$	 ** ** ** ** ** ** ** ** ** ** ** ** **

* Per cent.

(Main, R. K., and Schmidt, C. L. A., J. Gen. Physiol., 19, 127 (1935).)

similar to those which were obtained in the case of iron. The hydroxy-carboxylic acids markedly affect the color of the chloroform phase, whereas the unsubstituted acids exert a much smaller influence. The dicarboxylic acids, especially those with short

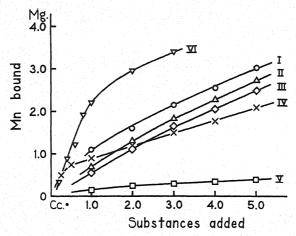


Fig. 19. Total manganese present 5.5 mg.; total volume of aqueous phase 20 cc.; volume of chloroform phase 15 cc.; Curve I, 0.0025 M thymus nucleic acid; Curve II, 1 per cent protamin sulfate; Curve III, 1 per cent edestin; Curve IV, 1 per cent gelatin; Curve V, 0.01 M proline; Curve VI, 1 per cent casein.

(Main, R. K., and Schmidt, C. L. A., J. Gen. Physiol., 19, 127 (1935).)

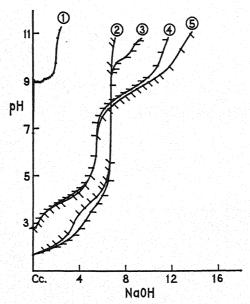


Fig. 20. Concentration of NaOH 0.1484 N; final total volume 22 cc.; Curve (1), 1 cc. 0.15 M MnCl₂; Curve (2), 5 cc. 0.1 M oxalic acid; Curve (3), 5 cc. 0.1 M oxalic acid +1 cc. 0.15 M MnCl₂; Curve (4), 10 cc. saturated solution 2-amino-phenol-4-sulfonic acid at 24°; Curve (5), 10 cc. saturated solution 2-amino-phenol-4-sulphonic acid +1 cc. 0.15 M MnCl₂. Temperature 24°.

(Main, R. K., and Schmidt, C. L. A., J. Gen. Physiol., 19, 127 (1935).)

chains, show a pronounced effect. Aspartic and glutamic acid show a decided influence, whereas the monocarboxylic amino acids influence the color in the chloroform phase but little. The proteins show evidence of forming complexes with manganous ions.

The second criterion for complex formation was based on migration experiments. The data are given in Table X. In solutions of aspartic and glutamic acid at pH 4.1 and 4.6, respectively, manganese is cationic, whereas at about 9.0, it is anionic. The direction of migration of casein also depends on the pH of the solution.

A third criterion for complex formation with manganese is the anomalous behavior of certain organic acids, when titrated with alkali, in the presence of manganous ions. This procedure was previously employed with magnesium by Zörkendörfer (31), and with iron and copper compounds by Smythe (32). The data relating to certain organic acids are shown in Fig. 20. The mixtures of organic acids and manganous chloride are more acid than the organic acid itself, indicating that the manganese-containing complex is a stronger acid than the original organic acid which was added.

On the basis of the Latimer and Porter (29) theory, divalent manganese probably forms a complex with hydroxy acids of the type

(a) (b) 0 (c) 0

$$H_3C - C - C$$
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Since the migration experiments indicate that manganese is cationic in the presence of an hydroxy acid, such as lactic acid, at pH 6.7, formula (a) or (b) is to be preferred. Since the negative charge on the carboxyl group at this pH is greater than the residual

charge on the hydroxyl group, it is not reasonable to assume that manganese would be attached to the hydroxy acid exclusively by means of the attraction of the residual negative charge on the hydroxyl group in preference to that of the strongly charged carboxyl group. If formula (c) were correct, then it would be expected that manganese would be anionic at pH 6.7.

Under the conditions of the experiments, a portion of the monocarboxylic amino acids exists in the classical form, and it is therefore not unexpected to find that these compounds show a slight tendency to form complexes with manganese. The following structural possibilities could exist in the case of the dicarboxylic acids:

Formulas (a) and (b) differ only in the degree of dissociation of carboxyl group (2). In solutions whose pH is greater than 6.0, formula (b) is the more plausible. Formula (c) is a special case of (b). Formula (d) represents a neutral molecule. It does not account for the fact that manganese is cationic at pH 5.9 and anionic at pH 9.1. The first three formulas are in agreement with the facts. If carboxyl group (2) ionizes, then manganese is anionic. If the pH of the solution is such as to repress the ionization of group (2), then the manganese which will migrate under the influence of a direct current is that which, on dissociation, will carry a positive charge.

The dicarboxylic amino acids react with manganous ions at pH 9.0 in a manner analogous to that of the unsubstituted dicarboxylic acids. The effect, as in the case of the latter compounds, depends upon the presence of the two free carboxyl groups in the molecule. In the case of hydroxy dicarboxylic acids, such as tartaric, the following possibilities exist:

Due to the proximity of the two carboxyl groups, the hydroxyl groups of this acid are more negative and more ionized than otherwise would be the case. Each hydroxyl group would not be expected to exhibit a negative charge comparable to that carried by an ionized carboxyl group; at least not to the extent as to make formula (c) preferable to (a). The migration data do not enable a differentiation to be made between formulas (a) and (b).

The above discussion is given in order to present the various factors which must be considered before a clear picture of the mode of combination which takes place between proteins and heavy metals can be obtained. The additional attraction which is necessary for complex formation between manganous ions and proteins is possibly furnished by the phenol group of tyrosine, when present, and the enol groups of the peptide linkages. The effect due to this attraction cannot be calculated quantitatively. Qualitatively, there is a correlation between the number of free carboxyl and phosphoric acid groups present in casein, edestin, and gelatin (see Fig. 19). Before a quantitative picture of this problem can be obtained, the structural relationships in the protein molecule will probably have to be known.

(3) Copper

Borsook and Thimann (33) have made use of absorption spectra as well as copper electrode measurements as criteria for the study of cupric complexes of glycine and alanine. They conclude that at least four types of complexes are formed between both glycine and alanine, and cupric ions. The type of compound formed is dependent upon the pH of the solution. A summary of the data is given in Table XI.

Table XI
Summary of Copper Complexes of Glycine and Alanine

Complex	Range of stability	Probable formula of complex	H ions set free per atom Cu bound	Absorption characteristics
1st acid glycine	Ca. pH 0.5-2.5	Cu glycine2	1	Curve similar to Cu- (OAc) ₂ in alcohol
2nd acid glycine	pH 2-5; in high dilution, up to pH 7	Cu ₂ glycine ₃	0.5	Peak just in infra- red
Neutral glycine	pH 5-8; overlapping basic complex to pH 10.5	Cu glycine2	1	Peak at 6250 Å.
Basic glycine	pH 8-12; not affected by dilution	Cu glycine2	2	Peak at 6700 Å.
1st acid alanine	Ca. pH 0.5-2.5	Cu alanine2	0	Very low absorption; about half that of 1st acid glycine and close to that of cupric ion
2nd acid ala- nine	pH 2.5-6; favored by dilution	Cu₂alanine₃	0.5	Exactly similar to 2nd acid glycine
Neutral alanine	pH 5-9; in dilute so- lutions to pH 11	Cu alanine ₃ or Cu ₂ ala- nine ₅	1	Peak at 6200 Å.; higher than neu- tral glycine
Basic alanine	pH 8-11; only in con- centrated solutions	Cu ala- nine≒₃	2	Peak at 6460 Å.; higher than basic glycine

(Borsook, H., and Thimann, K. V., J. Biol. Chem., 98, 671 (1932).)

The methods used for the estimation of the copper complexes present some interesting features. The copper electrode measurements will be considered first. The electrodes were prepared according to the directions given by Lewis and Lacey (34) and by

Getman (35). The general equation for the formation of any one of the complexes is

$$Cu \cdot A_m + rH^+ \rightleftharpoons Cu^{++} + m(AH)$$
 (10)

where $AH = \text{that form of the amino acid which takes part in the reaction, and <math>Cu \cdot A_m = \text{the complex}$. In this equation $Cu \cdot A_m$ bears a charge which varies with the complex under consideration and also contains (m-r)H atoms. These may be omitted from the mass law equation. The equilibrium is therefore

$$\frac{(\operatorname{Cu}^{++})(\operatorname{AH})^m}{(\operatorname{Cu} \cdot \operatorname{A}_m)(\operatorname{H}^+)^r} = K \tag{11}$$

where m=number of molecules of amino acids in the complex, and r=the number of hydrogen ions set free in the formation of one molecule of complex. For two different amino acid and hydrogen ion concentrations

$$\frac{(\mathrm{Cu}^{++})_1(\mathrm{AH})_1^m}{(\mathrm{Cu}\cdot\mathrm{A}_m)_1(\mathrm{H}^+)_1^r} = \frac{(\mathrm{Cu}^{++})_2\cdot(\mathrm{AH})_2^m}{(\mathrm{Cu}\cdot\mathrm{A}_m)_2\cdot(\mathrm{H}^+)_2^r}$$
(12)

Equation (12), on conversion to logarithms and on rearrangement, becomes

$$m \log \frac{(AH)_1}{(AH)_2} = \log \frac{(Cu^{++})_2}{(Cu^{++})_1} + \log \frac{(Cu \cdot A_m)_1}{(Cu \cdot A_m)_2} + r(pH_2 - pH_1)$$
(13)

The potential difference between copper electrodes in two solutions, where all other ions except the cupric ions are at practically the same concentration, is

$$E = \frac{RT}{nF} \ln \frac{(\text{Cu}^{++})_1}{(\text{Cu}^{++})_2}$$
 (14)

Converting to common logarithms and inserting in equation (13), the latter becomes

$$m \log \frac{(AH)_1}{(AH)_2} = (E_1 - E_2) \frac{nF}{2.303RT} + \log \frac{(Cu \cdot A_m)_1}{(Cu \cdot A_m)_2} + r(pH_2 - pH_1)$$
(15)

If practically the whole of the copper is in the complex form, the second term on the right-hand side of equation (15) can be omitted and the value of m (the number of molecules of amino acid combines with one cupric ion in a given complex) can be obtained from the potential difference between copper electrodes if the pH of the two solutions is the same. If an appreciable fraction of the

TABLE XII

Values of m, r, and K for $\frac{(Cu^{++}) (Glycine^{\pm})^m}{(CuGlycine_m) (H^+)^r} = K$ for First Acid Glycine

Concentration of $CuSO_4$ in all solutions = 0.02 M.

K	(11)	1.4 1.6 0.15 0.14 3.2 2.5
Values of M Log K (9) $r=1$	(10)	+0.15 1.4 +0.20 1.6 -0.83 0.15 -0.85 0.14 +0.51 3.2 +0.40 2.5
M	Log $r=0$ $r=1$ $r=2$	0.7 2.0 1.0
Jues of (9)	r=1	3.0 1.8 0.7 1.9 2.0 2.0 4.0 2.5 1.0 Mean value of M = 2.1
V8	r = 0	3.0 1.9 4.0 Me:
glycine [±] (8)	Log	-1.00 -0.83 -1.36 -0.93
Free		mole 0.10 0.149 0.088 0.044 0.117 0.1185
Total Ioniza- glycine tion of	gryeine (7)	per cent 20 30 44.8 45.4 45.4 29.2 46.3
Total glycine	(9)	mole 0.50 0.50 0.20 0.10 0.40
mplex	Log	-3.24 0.50 -2.94 0.50 -2.74 0.20 -2.85 0.10 -3.26 0.40 -2.85 0.40
Cu in complex (5)		mole mole 0.0014 S -2.85 0.00058 S -3.24 0.00084 S -3.08 0.00116 S -2.94 0.00020 -3.70 0.00180 -2.74 0.00059 -3.23 0.00141 -2.85 0.00145 -2.84 0.00055 -3.26 0.00058 -3.24 0.00142 -2.85
+	Log	1 3.28 1 3.28 1 3.28 1 3.28 1 3.24
Cu ⁺⁺ (4)		1.8* 0.0014 S 2.0* 0.00034 S 2.24 + 0.0206 0.00020 2.25 + 0.0067 0.00059 2.27 - 0.0070 0.00058
Cu electrode	e.m.f. (3)	volt +0.0206 +0.0067 -0.0048
μd	(3)	
Solu- tion	S S S	G 7 G 22 G 23 G 23 G 41 G 42

* = pH determined colorimetrically; error ±0.2.

t=pH determined by glass electrode; error ± 0.02 . S=from analysis of absorption spectra.

⁽Borsook, H., and Thimann, K. V., J. Biol. Chem., 98, 671 (1932).)

copper is not in the form of a complex, a correction is applied by assuming that the copper electrode potentials yield absolute Cu++ concentrations, and these values are deducted from those which represent the total copper which is present.

In acid solutions it was found that pH values great enough to suppress ionization of the carboxyl group of the amino acid also suppressed all complex formation. This indicates that, in the acid range, only the zwitterion form of these amino acids takes part in

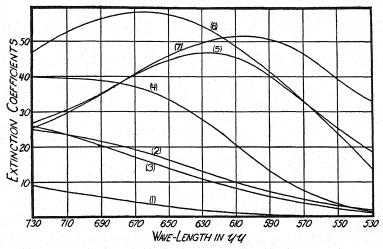


Fig. 21. Absorption curves of cupriglycine compounds. Extinction coefficients

 $\log \frac{I_0}{I} \cdot \frac{1}{c} \cdot \frac{1}{L(\text{em.})} \text{ at various wave-lengths in } \mu\mu. \text{ Curve 1, copper sulfate; Curve 2,}$

copper acetate in alcohol; Curve 3, first acid cupriglycine; Curve 4, second acid cupriglycine; Curve 5, neutral cupriglycine; Curve 6, basic cupriglycine; Curve 7, cupriammonium sulfate.

(Borsook, H., and Thimann, K. V., J. Biol. Chem., 98, 671 (1932).)

forming complexes with copper. Hence the concentration of free amino acid used in equation (15) refers only to that in the zwitterion form. This is the difference between the total calculated concentration of the zwitterion form of the amino acid at the pH of the solution and that bound in the complex. Where the concentration of amino acid is in excess of that of copper, and the pH between 4.5 and 8.0, no error is incurred by taking as the concentration of free amino acid the total amount initially added. The data for the first glycine complex are given in Table XII.

The absorption curves of the copper-glycine compounds are

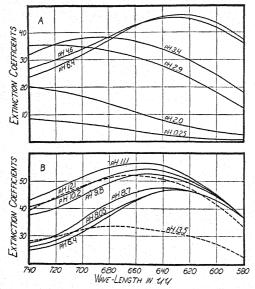


Fig. 22. A. Effect of changing H-ion concentration on the absorption of cupriglycine solutions containing the same amounts of copper and of glycine; Cu = 0.002 M, glycine = 0.50 M; acid range.

B. Same as A; alkaline range. Solutions in which precipitation occurred are shown in broken lines (pH 12.1 and 13.5).

(Borsook, H., and Thimann, K. V., J. Biol. Chem., 98, 671 (1932).)

represented in Figs. 21 and 22. The general method for solving such curves is as follows:

Let x =fraction of total copper as cupric ion

1-x = fraction of total copper in complex

 A_1 = absorption of cupric ion at wave-length λ_1

 B_1 =absorption of copper in complex form at wavelength λ_2

 $\Delta_1 = \text{total absorption of solution at wave-length } \lambda_1$ Then $xA_1 + (1-x)B_1 = \Delta_1$. Similarly, at another wave length, $xA_2 + (1-x)B_2 = \Delta_2$, etc.

Proceeding through the visible spectrum a value for x is obtained from the solutions of pairs of simultaneous equations. This will be constant throughout the spectrum if the absorption curves chosen, i.e., the values of A_1 , A_2 , A_3 , B_1 , B_2 , B_3 , etc., are correct. Values for A_1 , A_2 , A_3 , etc. were obtained from a pure copper sulfate solution at high dilution (see Curve 1, Fig. 21). If a constant value of x is obtained for any given set of values for B_1 , B_2 , B_3 , etc., the accuracy of these values for the absorption spectrum of the unknown compound is established.

Smythe (32) has concluded from anomalous titration data that cupric ions combine with tartaric acid to form a compound of the type,

$$\begin{array}{c|c} C \\ \hline \\ HC \\ \hline \\ C \\ \hline \\ O \\ \end{array} \begin{array}{c} O \\ OH_2 \\ \hline \\ O \\ \end{array} \begin{array}{c} O \\ Cu \\ Cu \\ \end{array}$$

(4) Donnan Equilibrium Measurements

Northrop and Kunitz (36) used the Donnan equilibrium as a criterion for studying the combination of isoelectric gelatin with a number of metallic ions. The total ion concentration in the pure salt solution outside the membrane and in the protein solution inside the membrane was measured. If the membrane potential is also measured, the ratio of the activity of the ion inside to that outside can be calculated and, if the activity coefficients are known, then the concentration of the ion inside can be calculated. The difference between this figure and the total concentration found by analysis gives the concentration of combined ion.

$$M_c = M_t - \frac{M_0 \gamma_0}{r \gamma_i} \tag{16}$$

where M_i =total concentration of ion found by analysis inside, M_c =concentration of combined ion, M_0 =total concentration of ion outside, γ_0 , γ_i =activity coefficients of the ion in the outside and inside solutions, respectively, and log r=0.4343 (Membrane Potential)/(RT/nF). In their experiments the total salt concentration differed only slightly between the inside and outside solution, so that, if the effect of the protein on the ionic strength of the solution is neglected, $\gamma_0 = \gamma_i$. The data of a typical experiment are given in Table XIII.

These workers conclude that the equivalent combining value of gelatin for Cu⁺⁺ is about 0.9 millimoles per gram and 0.4 to 0.5 millimoles per grams of deaminized gelatin. These values are the same as the equivalent combining power of this protein for hydrogen ions, indicating that the copper and hydrogen ions are attached to

the same group. The equivalent combining value of La⁺⁺⁺ and Al⁺⁺⁺ is about 0.5 millimoles per gram of gelatin. Deaminized gelatin has essentially the same value.

(5) Anomalous Osmosis

Under certain conditions, when a collodion bag filled with salt solution is placed in contact with water, instead of water moving inward as would be expected from the standpoint of osmotic pressure, it moves outward. This phenomenon is known as anomalous osmosis (see Chapter XIV). Loeb (37) showed experimentally

Table XIII

Donnan Equilibrium Measurements with Gelatin and AlCl₂ Gelatin,

5 per cent, $T = 37^{\circ}$, pH = 4.7

P.D.	Con	3.6:11: 1 01			
	Millimoles per	1000 gm. H ₂ O	Millimoles combined per	Millimoles Cl combined per gm. gelatin	
	Outside	Inside	gm. gelatin		
millivolts					
25.0	1.41	4.30	0.08	0.037	
18.0	3.90	8.75	0.16	0.065	
11.40	8.60	16.0	0.27	0.16	
4.90	30.4	41.0	0.47	0.24	
1.46	107.0	120.0	0.57	0.40	
1.80	74.3	86.7	0.52	0.40	
0.60	238.0	250.0	0.51	0.34	
0.72	276.0	287.0	0.62	0.16	
0.50	484.0	495.0	0.67	0.10	
0.24	445.7	457.3	0.44	0.40	
0.08	993.0	1006.0	0.45	0.60	

(Northrop, J. H., and Kunitz, M., J. Gen. Physiol., 11, 481 (1928).)

that when solutions of salts with a trivalent cation were separated from pure water by a protein-treated collodion membrane, water diffuses rapidly from the solvent into the solution, while no water diffuses into the solution when untreated membranes are employed. The anomalous behavior of trivalent ions is due to the formation of complex ions between the trivalent metal and the protein used to coat the membrane. Salts with trivalent cations, such as LaCl₃, form complex ions with proteins which are positively charged, while tetravalent anions, such as Fe(CN)²₆, yield complex ions which are charged negatively.

(6) Cystine and Cysteine Complexes

In the above examples of proteins which form complexes with the heavy metals, the cystine and cysteine content were not considered. The content of these amino acids in the proteins used (casein and gelatin) is low, and it was therefore assumed that the chief factors in the formation of complexes are groups other than the -S-S- or -SH groups. A number of studies have been carried out which, however, show that cystine and cysteine form complexes with heavy metals. Thus Michaelis (38) has shown, on the basis of oxidation-reduction reactions, that cobaltous salts, at pH 7 to 8, form complexes with cysteine, among which cobaltotricysteine is particularly prevalent. This compound is oxidized in two steps, the first leading to cobaltitricysteine, the second step leading to cobalto-cysteine-cystine which is stable. The ferrocysteine-cystine complex reacts with two molecules of free cysteine so as to furnish free cystine and ferrotricysteine. This involves a cyclic process which ends in the oxidation of all of the cysteine to cystine. When oxygen gas is the oxidant, it is completely reduced to water. The reaction proceeds in two steps. In the first step hydrogen peroxide is formed and in the second step water. The complex of cysteine with nickel cannot be oxidized by oxygen. Nickel is not easily oxidized to the nickelic state. The cobalt and iron complexes of cystine and cysteine are schematically represented in Fig. 23.

Further studies on this subject have been carried out by Schubert (39). The types of compounds which he believes are formed with cobalt are shown in Fig. 24. When cystine is treated with either HgSO₄ or Ag₂SO₄ in the presence of dilute sulfuric acid, it is extensively reduced to cysteine with some oxidation to cysteic acid according to the equation (40),

$$2R-S-S-R+H_2O\rightarrow R-SO_3-H+5RSH.$$

(7) Other Heavy Metal Complexes

Mercuric chloride in the presence of alkali is used as a precipitant for amino acids. This is due to the formation of mercury-containing complexes. According to Vickery and Gordon (41), the composition of the precipitates is that which is given in Table XIV. Bergmann and Fox (42) have obtained the compound [Cr(C₂O₄)₃]₉K₁₈-(C₂H₆O₂N)₉·HCl, when glycine, in excess, is treated with potassium trioxalatochromiate in the presence of hydrochloric acid in wateralcohol solution. When glycine is not present in excess, a product

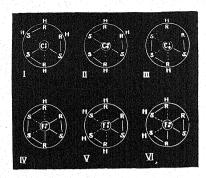


Fig. 23. Compounds of Cobalt and Iron with Cystine or Cysteine.

R represents CH₂·CHNH₂COO. Cystine is, therefore, HSRH; cystine is HRSSRH. Main valences are represented by solid lines; residual valences by dash lines.

Formula I. Cobaltous tricysteine. The two valences are attached to S groups, not to R groups. The asymmetry with respect to the spacial distribution of the two valences is only apparent and disappears when the plane figure is substituted by the octahedral distribution of the six coordination places around the central metal atom, according to Werner. Instead, other possibilities of isomerism arise. Ferrous tricysteine must be considered analogous to this complex.

Formula II. Cobaltic tricysteine. This arises from Formula I by loss of 1 H atom. The third valence is attached again to S, not to R. Ferric tricysteine is analogous.

Formula III. Cobaltous cysteine-cystine. This arises from Formula II by loss of another H atom. One valence is attached to S, the second, however, necessarily to R because no SH group is available. Therefore this complex cannot undergo a further dehydrogenation and must remain in the cobaltous state. Ferrous cysteine-cystine is analogous to this complex, but here further dehydrogenation is possible, which would produce Formula IV.

Formula IV. Ferric cysteine-cystine. When in ferrous cysteine-cystine (Formula III with Fe instead of Co) 1 molecule of cystine is replaced by 2 molecules of cysteine, there arises Formula V.

Formula V. Ferrous tricysteine. This differs from the structure represented by Formula I (with Fe instead of Co) only by the distribution of the main valences. On account of the greater affinity of iron for S than for R, Formula V will shift spontaneously to Formula I and the cycle is closed.

In case that Formula III is oxidized to Formula IV before an exchange of 1 cystine molecule for 2 of cysteine can take place, this exchange will occur in Formula IV instead of in Formula III. This leads to Formula VI, and then the rearrangement of the three main valences in such a way that they become attached to S instead of R, resulting in Formula II (with Fe instead of Co) takes place. A cycle is closed in this way also.

(Michaelis, L., J. Biol. Chem., 84, 777 (1929).)

having the composition $[Cr(C_2O_4)_3]_6K_{13}(C_2H_6O_2N)_5\cdot 3H_2O$ crystallizes out of solution. Potassium trioxalatoferriate and potassium trioxalatocobaltiate form analogous compounds with glycine.

Lack of space prevents discussion of all of the compounds which are formed between heavy metals and amino acids or proteins.

* The suggestion that this may be a double internal complex of the following formula will be considered in a later paper. See reference (39).

** As this compound is hydrated, it may be that the normal six coördination places of the trivalent cobalt are completed by the addition of two molecules of water.

Fig. 24 (Schubert, M. P., J. Amer. Chem. Soc., 53, 3851 (1931).)

The following references (besides those already given) will be of help to the reader in gaining access to the literature: aluminum (43); cerium (44); gold (45); chromium (43); nickel (43); zinc (46);

Table XIV

Composition in Terms of Atomic Ratios of Precipitates that Contain Complex Mercuric

Chloride Compounds of Amino Acids

(The atomic ratio of nitrogen is taken arbitrarily as 2)

	Mercury	Chlorine	Alkali	
Barium glycine	3.10	1.24	0.44	
Barium alanine	3.08	1.43	0.71	
Barium leucine	3.08	1.45	0.72	
Barium phenylalanine	3.02	1.66	0.44	
Barium glutamic acid	3.36	1.05	1.30	
Sodium glycine	3.23	1.07	1.12	
Lithium glycine	3.25	0.84	0.78	
Sodium alanine	3.27	1.02	1.01	
Barium lysine	2.21	1.53	0.02	

lead (47); cobalt (43, 46, 48); and silver (45, 46, 49). Much of the literature is given by Lieben and Lowe (50).

3. COMPOUNDS OF AMINO ACIDS AND PROTEINS WITH OTHER SUBSTANCES

The amphoteric nature of the amino acids and proteins makes it possible for them to combine with a variety of compounds which

TITRATION CURVES

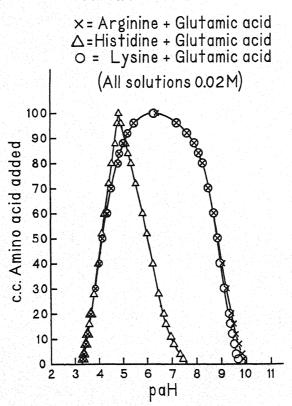


Fig. 25 (Miyamoto, S., and Schmidt, C. L. A., J. Biol. Chem., 87, 327 (1930).)

are acidic or basic. Thus, protamin edestinate was prepared by Schmidt (51), protamin caseinate by Gay and Robertson (52), and globin caseinate by Robertson (53). The more recent preparation of protamin-insulinate (54) is another example of this type. Under proper conditions of acidity, dicarboxylic amino acids will unite with the basic amino acids to form compound amino acids

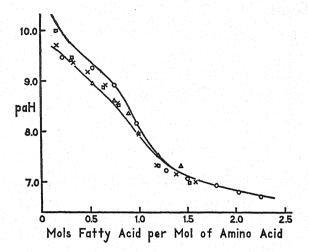


Fig. 26. Titration curves of lysine and arginine with lauric and oleic acids. The upper curve is calculated for the titration of arginine with lauric or oleic acids. The lower curve is calculated for the titration of lysine with lauric or oleic acids. The points represent experimental values. O, lysine and lauric acid; Δ , lysine and oleic acid; \Box , arginine and lauric acid; \times , arginine and oleic acid.

(Jukes, T. H., and Schmidt, C. L. A., J. Biol. Chem., 110, 9 (1935).)

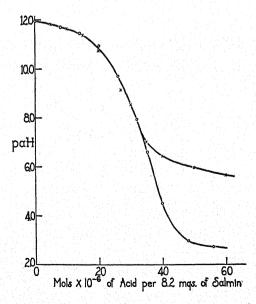


Fig. 27. Titration curves of salmin with hydrochloric acid O and with caprylic acid X in 30 per cent ethanol. The points indicate experimental observations, and the curves have been plotted to fit the points.

(Jukes, T. H., and Schmidt, C. L. A., J. Biol. Chem., 110, 9 (1935).)

nucleic acid.

(55). Fig. 25 illustrates the formation of compounds between the hexone bases and glutamic acid. The salt formation which takes place between two amino acids or proteins can be extended to all such compounds which have differences in dissociation constants. The union of toxin and antitoxin may possibly be like the formation of such compounds.

Amino acids and proteins may also form salts with fatty acids. Compounds of this type have been prepared by Jukes and Schmidt (56) (see Figs. 26 and 27). On the other hand, proteins may combine with fatty acids in an ester linkage. Compounds of this type

Table XV Carbohudrate Content of Proteins

[마마마 : 4 : 1 : 1 : 1 : 1 : 1 : 1 : 1 : 1 : 1	
Ovalbumin	1.7 per cent; mannose
Easily soluble serum-albumin fraction	0.47 per cent; mannose + galactose
Sparingly soluble serum-albumin fraction	0.02 per cent
Horse-serum globulin	1.82 per cent; mannose + galactose.
Casein	0.31 per cent; galactose, no lactose
Lactalbumin	0.44 per cent; galactose, no lactose
Sparingly soluble wheat-gliadin fraction.	0.20 per cent; mannose
Egg-white proteins:	
globulin	4.0 per cent; mannose
albumin	1.7 per cent; mannose
conalbumin	2.8 per cent; 3 mannose +1 galactose
mucin	14.9 per cent; mannose + galactose
mucoid	9.2 per cent; 3 mannose +1 galactose
(Rimington, C., Ann. Rev. Biochem., S	stanford University (Calif.) 1936, 5, p.
138.)	토론 역소 본 기를 가능히 하는 것이다.

have been synthesized by means of the Schotten-Baumann reaction by Bang (57), and by Izar and his co-workers (58). Phosphoric acid is combined in proteins as an ester (59). The nucleo-proteins are compounds of basic proteins with nucleic acid. Such compounds have been studied by Milroy and others (54, 60, 61). Arsenic may replace phosphorus and form compounds analogous to

Within the last few years it has become increasingly evident that carbohydrate groups are an integral part of a variety of proteins (62). The compilation given in Table XV was prepared by Rimington (63). Proteins may contain glucosamine combined with other sugars, such as glucosamino-dimannose and glucosamino-galactose, which, in certain cases, may be present together in the same molecule. Both silk fibroin and silk peptone appear to form complexes with amylose (64). Przylecki and his co-workers (65)

have attempted to study the mode of combination of carbohydrates, especially those which are combined with phosphoric acid, and proteins. They consider that secondary valence forces may play an important rôle. As shown by Heidelberger, the carbohydrate-containing proteins are of great importance in immunology (see Chapter XVIII).

It is altogether probable that such precipitating agents as picric acid, flavianic acid, rhodanilic acid, Reinecke salt, and other alkaloidal reagents form definite compounds with amino acids and with proteins (66). Atropine, quinine, strychnine, guanidine, and epinephrine combine with proteins on the alkaline side of the isoelectric point (67).

The fact that amino acids and proteins can combine with a large variety of elements and compounds is of great importance in biological phenomena. When one considers the complexity of biological fluids and tissues, it is apparent that the elucidation of this subject is far from being a closed chapter. Hardy (68) and Robertson (69) long ago hinted that large protein complexes may play important rôles in biological phenomena.

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CHAPTER XIV

MEMBRANE EQUILIBRIA

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1. THE DONNAN EQUILIBRIUM

In 1911, F. G. Donnan (1) published his theory of membrane equilibrium. It was destined to furnish the guiding principle for the elucidation of many heretofore puzzling problems in connection with the physical chemistry of the proteins. The theory was first applied to proteins in 1914 by Proctor (2) in connection with the swelling of gelatin, but it remained for Jacques Loeb (3) to show the great importance of this theory in accounting for the influence of acids, bases, and neutral salts of varying valency on the membrane potentials, osmotic pressure, swelling of gels, and on a special type of viscosity of protein systems. On the basis of the Donnan membrane distribution, Loeb developed his theory of the colloidal behavior of the proteins.

The theory of membrane equilibrium applies whenever one ionic species, usually a colloidal ion, in a mixture of electrolytes, is prevented from diffusing freely to all parts of the system by any mechanism which puts a constraint upon the ionic species in question. Most commonly the constraint results from the presence of a dividing membrane to which the colloidal ion is impermeable, or because the ion is part of a fixed structure such as part of a gel.

Donnan showed that in a system of electrolytes separated into two parts by a membrane to which one of the ionic species is impermeable, there will, in general, be an unequal distribution of the diffusible ions between the solutions on the two sides of the membrane. As a consequence of this unequal distribution of electrolytes, a difference in electrical potential must exist between the solutions on the two sides of the membrane, the magnitude of which, in accordance with the Nernst equation, is proportional to the logarithm of the ionic ratios. Also, as a further consequence of the unequal ionic distribution, there must exist a difference in osmotic pressure between the solutions on opposite sides of the

membrane. The differences in electrical potential and osmotic pressure form the basis for explaining the special properties of electrolytes on protein systems already mentioned; namely, membrane potentials, osmotic pressure, swelling in gels, a certain type of viscosity, and the effect of salts of different valency on these properties.

In the treatment that is to follow, the mathematical equations for the ion distribution in systems containing electrolytes of differing valency will first be developed, and the theory and experimental treatment of each one of the special properties just mentioned will then be taken up.

Let us first consider the simplest kind of a system that can be used; i.e., a solution of a protein salt yielding the ions P⁺ and Cl⁻ in a vessel, which is separated by a membrane, M, from a solution of hydrogen chloride as is illustrated on the left of Fig. 1, in order

Fig. 1

to show how the basic equations are derived according to the method used by Donnan. The equivalent concentration of the protein salt is denoted by Z. Since, in this system, all the electrolytes are freely diffusible except the colloidal protein ion P+, the hydrochloric acid will diffuse from (2) to (1) until equilibrium is established. At equilibrium the concentrations which are obtained are represented by the letters opposite each ionic species in the diagram in the right of Fig. 1. The rigorous condition for the establishment of equilibrium between the two sides of the membrane is that the product of the activity of hydrogen and chloride ion on the side (1) becomes equal to the hydrogen and chloride ion activity product on the side (2). In the equilibrium state the free energy change, $(\Delta F)_{P.T.}$, equals zero. From this it follows that the change in free energy required to transport reversibly and isothermally dn moles of H+ from (2) to (1) is equivalent to the energy gained by a similar transport of dn moles of Cl- from (2) to (1). From this the free energy change is given by the equation.

$$(dF)_{\tt P.T.}\!=\!RT \ {\rm dn} \ {\rm ln} \ \frac{[{\rm H}^+]_2}{[{\rm H}^+]_1}\!\!+\!RT \ {\rm dn} \ {\rm ln} \ \frac{[{\rm Cl}^-]_2}{[{\rm Cl}^-]_1}\!=\!0$$

It is quite readily seen then that

$$\frac{[H^{+}]_{2}}{[H^{+}]_{1}} = \frac{[Cl^{-}]_{1}}{[Cl^{-}]_{2}} \quad \text{or} \quad [H^{+}]_{2}[Cl^{-}]_{2} = [H^{+}]_{1}[Cl^{-}]_{1}$$
 (1)

Or, if the symbols shown in the diagram on the right of Fig. 1 are used to represent the ionic concentrations at equilibrium, there is obtained

$$\frac{X}{Y} = \frac{Y+Z}{X}, \quad \text{or} \quad X^2 = Y(Y+Z)$$
 (2)

The ratio, $[H^+]_2/[H^+]_1 = [Cl^-]_1/[Cl^-]_2 = X/Y$, is very often designated by λ .

The derivation given is only strictly valid when the terms used are the activities and not the concentrations of the ions. For our

Initial Concentrations Equilibrium Concentrations P Cl = ZHCl P Cl in H⁺ in (2) = X | H⁺ in (1) = Y | $\lambda = \frac{X}{V}$ added to (1)(2)N N 0.00083 0.10 0.01 0.10 0.00917 11.05 0.10 0.05 0.10 0.03750.01253.0 0.100.10 0.100.06670.03332.00.10 1.00 0.10 0.5230.4771.1

TABLE I

present purpose, the use of concentrations will be a sufficiently close approximation to the experimental requirements. A more rigorous derivation for concentration in terms in which the activity coefficients of the ions in the system are introduced, has been developed by Hückel (4).

Equation (1) shows that the product of the concentration of hydrogen and chloride ions on the two sides of the membrane is equal and this can only be true if

$$[H^+]_1 < [H^+]_2;$$
 and $[Cl^-]_1 > [Cl^-]_2$ (3)

or, using the letters given in equation (2),

$$X > Y$$
 and $(Y+Z) > X$ (3a)

To illustrate numerically the ion concentration gradients that may result from a membrane equilibrium such as is postulated in Fig. 1, the differences in concentration for hydrogen chloride between the two sides of the membrane are calculated in Table I for a system where the concentration of protein chloride is kept constant at 0.1 N and the hydrogen chloride which was initially introduced is varied. In making the calculations, it was assumed that there are equal volumes of solution on each side of the membrane, and that they remain unchanged throughout.

From the table it is seen that when the concentration of hydrogen chloride added is small as compared to the concentration of the protein chloride, only a small proportion of the acid can get into

$$Z = P^{+}$$

$$Y = H^{+}$$

$$\frac{Y}{2} + \frac{Z}{2} = SO_{4}^{-}$$

$$SO_{4}^{-} = \frac{X}{2}$$

$$(1)$$
Fig. 2

the side containing the protein chloride, and, as the amount of hydrogen chloride added is increased, the gradient becomes smaller and smaller.

When, instead of a uni-univalent electrolyte, there is introduced an electrolyte of the type of sulfuric acid, the equilibrium distribution shown in Fig. 2 is obtained. The distribution equation for this system is

$$\frac{[H^{+}]_{2}}{[H^{+}]_{1}} = \sqrt{\frac{[SO_{4}^{-}]_{1}}{[SO_{4}^{-}]_{2}}}$$
 (4)

or

$$X^3 = Y^2(Y+Z) \tag{5}$$

The concentration units of X, Y, and Z are here expressed in equivalents per 1000 gm. of water. Equation (5) shows that a change in the valence of a diffusible ion results in a change of the degree of the algebraic equation expressing the distribution.

From the preceding treatment there can be generalized the distribution which results in a system containing the non-diffusible protein ion, P⁺, and any number of diffusible ions. The distribution

equation for such a case is expressed by the equation,

$$\lambda = \frac{[H^{+}]_{2}}{[H^{+}]_{1}} = \frac{[K^{+}]_{2}}{[K^{+}]_{1}} = \sqrt{\frac{[Ca^{++}]_{2}}{[Ca^{++}]_{1}}} = \sqrt[n]{\frac{[B^{n+}]_{2}}{[B^{n+}]_{1}}} = \frac{[OH^{-}]_{1}}{[OH^{-}]_{2}}$$

$$= \frac{[Cl^{-}]_{1}}{[Cl^{-}]_{2}} = \sqrt[n]{\frac{[SO_{4}^{-}]_{1}}{[SO_{4}^{-}]_{2}}} = \sqrt[n]{\frac{[A^{m-}]_{1}}{[A^{m-}]_{2}}} \tag{6}$$

In equation (6), λ represents the Donnan distribution ratio, B a cation of valency n, and A an anion of valency m. The other terms require no further explanation. It follows, furthermore, from the principle of proportionality, that equation (6) can be transformed to

$$\lambda = \frac{[H^{+}]_{2} + [K^{+}]_{2} + \sqrt{[Ca^{++}]_{2} + \sqrt{[B^{n+}]_{2}}}}{[H^{+}]_{1} + [K^{+}]_{1} + \sqrt{[Ca^{++}]_{1} + \sqrt{[B^{n+}]_{1}}}}$$

$$= \frac{[OH^{-}]_{1} + [Cl^{-}]_{1} + \sqrt{[SO_{4}^{-}]_{1} + \sqrt{[A^{m-}]_{1}}}}{[OH^{-}]_{2} + [Cl^{-}]_{2} + \sqrt{[SO_{4}^{-}]_{2} + \sqrt{[A^{m-}]_{2}}}}$$
(7)

or other equations of a similar type.

So far, we have carried out the development as if the protein salt were the salt of a strong base whose ionization is not affected by changes in the acidity. Since the proteins are amphoteric and exhibit in solution the properties of either a weak base or a weak acid, depending on whether the solution is at a pH more acid or more alkaline than the isoelectric point, a different mode of treatment has to be used to give the equations for the membrane equilibria of protein systems that take into account the amphoteric properties of proteins. Accordingly, there will now be developed the membrane equilibrium equations, first for a non-diffusible weak base, then for a corresponding weak acid, and finally, for a non-diffusible amphoteric electrolyte. The method of deriving these equations will largely follow the procedure published by Hitchcock (5).

For the sake of simplicity, consider an amphoteric substance (protein) with but one dissociable basic and one dissociable acidic group. Let this protein be represented by the symbol HPOH, and its ionization by the following equations:

HPOH
$$\rightleftharpoons$$
HP++OH-;
$$\frac{[\text{HP+}] \times [\text{OH-}]}{[\text{HPOH}]} = k_b$$
 (8)

HPOH
$$\rightleftharpoons$$
H⁺+POH⁻;
$$\frac{[H^+] \times [POH^-]}{[HPOH]} = k_a$$
 (9)

The equation governing the dissociation of water,

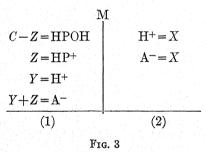
$$[H^+] \times [OH^-] = K_w$$

will need to be employed also.

From these ionization equations we can now develop the membrane distribution equations just mentioned.

2. MEMBRANE EQUILIBRIUM EQUATIONS FOR A SYSTEM CONTAINING A NON-DIFFUSIBLE BASE AND A MONOVALENT ACID

Let us consider first that the protein is on the acid side of its isoelectric point and is behaving only as a weak base. Also, the only diffusible electrolyte in the system consists of a strong monovalent acid which is symbolized by the letters HA. The equilibrium distribution obtained is shown in Fig. 3.



In Fig. 3, the letter opposite each chemical species represents the concentration of the chemical species. The concentration of the total protein in all of its forms is represented by C. From the equilibrium distribution in Fig. 3, it is seen that equation (8) becomes

$$\frac{Z \times K_w}{(C-Z)Y} = k_b$$

when $[OH^-]$ is replaced by K_w/Y . Now let K_1 represent K_w/k_b . Then,

 $ZK_1 = (C - Z)Y$ $ZK_1 + ZY = CY$

and

$$Z = \frac{CY}{K_1 + Y} \tag{10}$$

Substituting (10) in the Donnan distribution equation $X^2 = Y(Y+Z)$ there is obtained

$$X^2 = Y\left(Y + \frac{CY}{K_1 + Y}\right)$$
 or $X = Y\sqrt{1 + \frac{C}{K_1 + Y}}$

From this the Donnan ratio becomes

$$\lambda = \frac{X}{Y} = \sqrt{1 + \frac{C}{K_1 + Y}} \tag{11}$$

From equation (11) it is apparent that, as the concentration of hydrogen ions is increased, the value of the Donnan ratio, λ , must always decrease and finally approach unity as a limit.

3. MEMBRANE EQUILIBRIUM EQUATIONS FOR A SYSTEM CONTAINING A NON-DIFFUSIBLE ACID AND A DIFFUSIBLE MONOVALENT BASE

Let us next consider that the protein is on the alkaline side of its isoelectric point and is behaving only as a non-diffusible weak acid. The diffusible electrolyte in this instance consists of a strong monovalent base represented by B(OH). The equilibrium concentrations that result for this condition are shown in the diagram given by Fig. 4.

$$C-W = \text{HPOH}$$

$$W = \text{POH}^-$$

$$V = \text{OH}^-$$

$$V+W = \text{B}^+$$

$$(1)$$

$$(2)$$

Fig. 4

The concentration of all of the protein species is again denoted by C and the symbols for all chemical species are given by the letters shown in the figure.

Now if, in equation (9), we substitute K_w/V for [H+], W for [POH-], and C-W for [HPOH], there is obtained

$$\frac{W \times K_w}{(C - W)V} = k_a$$

Let

$$\frac{K_w}{k} = K_2$$

Then

$$WK_2 = V(C - W) \qquad \text{and}$$

$$W = \frac{VC}{K_2 + V} \tag{12}$$

The membrane distribution is given by the equation

$$U^2 = V(V + W)$$

Substituting equation (12) for the value of W, there is obtained

$$U = V \sqrt{1 + \frac{C}{K_2 + V}} \tag{13}$$

From this we have for the Donnan ratio,

$$\lambda = \frac{V}{U} = \frac{1}{\sqrt{1 + \frac{C}{K_2 + V}}} \tag{14}$$

Equation (14) shows that the Donnan ratio on the alkaline side of the isoelectric point, as it has been defined by equation (6), is less than unity, but, as the alkalinity is increased, λ increases and approaches the value of unity as a limit.

4. MEMBRANE EQUILIBRIUM EQUATIONS FOR A SYSTEM CONTAINING A NON-DIFFUSIBLE AMPHOTERIC ELECTROLYTE AND A DIFFUSIBLE MONOVALENT ACID

Lastly, we need to consider that the protein is in solution at a pH not far removed from the isoelectric point so that the amphoteric properties can not be neglected. The equilibrium distribution for this condition is given by Fig. 5 when the diffusible electrolyte

$$C-W-Z=HPOH & H^{+}=X \\ Z=HP^{+} & OH^{-}=U \\ W=POH^{-} & A^{-}=X-U \\ Y=H^{+} \\ V=OH^{-} \\ (Y+Z-W-V)=A^{-} \\ (1) & (2)$$

Fig. 5

is taken to be the acid HA. As before, the concentrations are indicated by the letters opposite the chemical species. The equilibrium distribution can be written as

$$\lambda = \frac{X}{Y} = \frac{V}{U} = \frac{Y + Z - W - V}{X - U} \tag{15}$$

Equations (8) and (9), from the distribution given in Fig. 5, take the form,

$$\frac{VZ}{C - W - Z} = k_b \tag{16}$$

and

$$\frac{YW}{C - W - Z} = k_a \tag{16a}$$

Since $X = \lambda Y$ and $U = V/\lambda$, we can substitute these values in equation (15):

$$\lambda = \frac{Y + Z - W - V}{\lambda Y - \frac{V}{\lambda}}$$

from which there is obtained

$$\lambda^2 Y - V = Y + Z - W - V$$

On rearranging this we have

$$Z - W = Y(\lambda^2 - 1) \tag{17}$$

Next, by simultaneously solving equations (16) and (16a) for W and Z, we have

$$W = \frac{CK_w k_a}{Y^2 k_b + YK_w + K_w k_a} \tag{18}$$

and

$$Z = \frac{CY^2k_b}{Y^2k_b + YK_w + K_wk_a} \tag{18a}$$

Subtracting (18) from (18a) there is obtained

$$\frac{CY^{2}k_{b} - CK_{w}k_{a}}{Y^{2}k_{b} + YK_{w} + K_{w}k_{a}} = Y(\lambda^{2} - 1)$$
(19)

Now dividing through by k_b the equation becomes

$$\frac{CY^{2}-C\frac{k_{a}}{k_{b}}K_{w}}{Y^{2}+Y\frac{K_{w}}{k_{b}}+\frac{K_{a}}{k_{b}}K_{w}}=Y(\lambda^{2}-1)$$
(19a)

Since $\sqrt{(k_a/k_b)K_w}$ is the constant of the isoelectric point commonly represented by I,* and K_w/k_b has already been given the symbol K_1 , we can substitute these terms in equation (19a).

$$\frac{C(Y^2 - I^2)}{Y^2 + YK_1 + I^2} = Y(\lambda^2 - 1) \tag{20}$$

From this, solving for λ , there is obtained the equation,

$$\lambda = \sqrt{1 + \frac{C(Y^2 - I^2)}{Y(Y^2 + YK_1 + I^2)}} \tag{21}$$

Equation (21) gives the membrane distribution ratio in terms of the hydrogen ion concentration of the protein-containing solution and of the isoelectric point of the protein. It is to be noted from equation (21) that, at the isoelectric point, since Y = I, the term under the square root sign reduces to 1 and thus $\lambda = 1$. In other words, the Donnan effect at the isoelectric point is zero. On the acid side of the isoelectric point Y > I and so λ at first must be greater than 1. Now with a large excess of acid where I^2 becomes negligible as compared to Y^2 , the equation reduces to

$$\lambda = \sqrt{1 + \frac{C}{V + K_1}}$$

which, it is seen, is equation (11). From this, as has been pointed out for equation (11), as Y becomes greatly increased, λ again decreases and approaches the limiting value of 1. Similarly, equation (21) shows that on changing the reaction to the alkaline side of the isoelectric point, λ becomes less than 1, which simply means, as would be expected, that since the protein changes in sign from positive to negative, the Donnan ratio is reversed. Again, as the solution becomes more and more alkaline, it can be shown that equation (21) becomes equivalent to equation (14) and, as has been pointed out for equation (14), λ in the limit again approaches the value of 1.

^{*} A discussion of certain alternative definitions and the general theory of the isoelectric point of amphoteric electrolytes is given in Chapter XI.

Now that the development of the membrane equilibrium equations for the various conditions has been completed, we shall turn to the consideration of the physical properties that are influenced by the Donnan equilibrium.

5. MEMBRANE POTENTIALS

We have seen that in the system represented by Fig. 1 (containing protein chloride and hydrogen chloride on side (1) of the membrane and hydrogen chloride only on side (2)) when equilibrium is established, $[H^+]_2 > [H^+]_1$. From this we know that if solutions (1) and (2) were used for the electrolyte solutions in hydrogen electrode half cells and the two half cells were connected in such a way that there is no liquid junction, there would be established a potential difference whose value would be given by equation (22),

 $E = \frac{RT}{F} \ln \frac{[H^+]_2}{[H^+]_1}$ (22)

In this equation E is the electromotive force in volts, R is the gas law constant (8.316 joules per degree), T is the absolute temperature, F is the Faraday constant (96,500 coulombs), and I represents natural logarithms.

On the other hand, if the hydrogen electrodes were introduced on the two sides of the membrane when equilibrium has been established, we know from thermodynamic considerations that the potential difference must be zero. The significance of this is that there must be a potential on the membrane equal in magnitude but opposite in sign to the potential difference of the concentration cell that would be obtained from the two solutions where no membrane is interposed. The membrane potential then arises from the tendency of the H⁺ to diffuse from (2) to (1) and conversely the tendency of the Cl⁻ to diffuse from (1) to (2). Just as the membrane potential is determined by the ratio of [H⁺]₂: [H⁺]₁, it follows from equation (1) that it is also determined by the ratio [Cl⁻]₁: [Cl⁻]₂. Since, as is pointed out in equation (6),

$$\frac{[H^+]_2}{[H^+]_1} = \frac{[Cl^-]_1}{[Cl^-]_2} = \lambda$$

we have, in general,

$$E = \frac{RT}{F} \ln \lambda \tag{23}$$

In this equation any terms can be substituted for λ which are equivalent to the Donnan ratio.

Numerically, if the measurements are made in volts at 25°, and logarithms to the base 10 are employed instead of natural logarithms, equation (22) becomes

$$E = 0.05915 \log \lambda = 0.05915 \log \frac{[H^+]_2}{[H^+]_1}$$
 (24)

The very common unit of hydrogen ion concentration or hydrogen ion activity employed in biological work is the pH term of

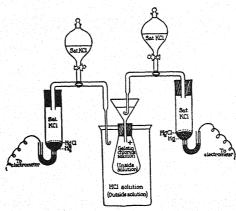


Fig. 6. Method of measuring the P.D. between gelatin chloride solution in a collodion bag and the outside HCl solution in beaker.

(Loeb, J., J. Gen. Physiol., 3, 667 (1920-21); Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 178.)

Sörensen. Since this is defined as the negative logarithm of the hydrogen ion concentration, equation (24) can be changed to

$$E = 0.05915(pH_1 - pH_2)$$
 (25)

From this equation the membrane potential is seen to be proportional to the difference between the pH of the protein-containing and the protein-free solutions.

The membrane potential can be readily calculated from the hydrogen electrode values of the solutions on the two sides of the membrane. However, this is not the only means of directly determining the membrane potential. A very ingenious method of measuring the potentials directly was devised by J. Loeb. This is illustrated in Fig. 6. In the method as used by Loeb, a sample of protein in acid solution was introduced into a collodion bag of

about 50 cc. capacity. The bag was closed with a rubber stopper through which there extended a glass tube to serve as a manometer. The bag was then submerged in a beaker containing 350 cc. of a solution of the same concentration of acid as was used to make up the protein solution. After a period of about 24 hours, in order to allow the attainment of osmotic equilibrium, the glass tube was replaced by a funnel and the membrane potential was measured by the set-up shown in Fig. 6. The E.M.F. that is measured is given by the diagram:

In the measurement of the potential of these cells, Loeb found that the protein-containing solution inside of the bag had a positive

Table II

Comparison Between the Directly Measured Membrane Potentials and the Potentials

Calculated from the pH of the Solutions in Gelatin Chloride Solutions

Equilibrated with Varying Amounts of Hydrochloric Acid

	Hydrochloric acid added to 100 cc. of 1 per cent isoelectric gelatin in cc. of 0.1 N							
	1	2	4	6	8	10	15	20
ΔpH. Membrane potential	0.42	0.53	0.59	0.59	0.46	0.44	0.24	0.13
calculated from pH (millivolts) Measured mem-	24.7	31.0	34.5	34.5	27.0	25.8	14.0	7.6
brane potential (millivolts)	24.0	32.0	33.0	32.5	26.0	24.5	11.2	6.4

(Loeb, J., Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 183.)

charge, while the outside solution, with no protein, was negatively charged. This is the reverse of the direction of the current that is obtained according to equation (24) but is the correct sign for the membrane potential. Just why this particular set-up of Loeb's measures the membrane potential, as it actually does, has never been satisfactorily explained from a theoretical standpoint. It is not the equilibrium state which is measured, for we know that the equilibrium state gives a potential of zero. The likelihood seems to

be that the potential in Loeb's method is determined by differences in diffusion potentials, chiefly of the hydrogen ion, between side (1) and side (2) against the saturated KCl of the calomel electrodes.

Many determinations of the membrane potentials of protein systems by the method just described have been carried out by

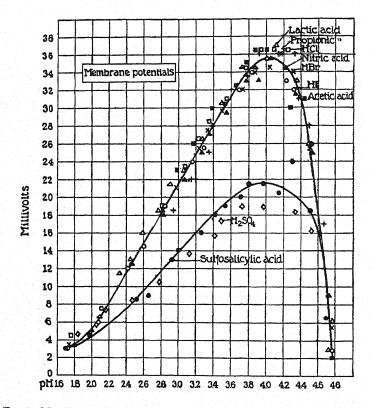


Fig. 7. Membrane potentials of gelatin dissolved in solutions of dilute univalent and bivalent acids.

(Loeb, J., and Kunitz, M., J. Gen. Physiol., 5, 665 (1922-23); Loeb, J., Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 188.)

Loeb and his co-workers. Proteins that have been studied are gelatin, egg albumin, edestin, and serum globulin (6, 7, 8, 9). In a large number of instances the figures obtained for the directly measured membrane potentials have been checked by values calculated from the pH of the solutions on the two sides of the membrane, as determined by the hydrogen electrode, and good

agreement was obtained. An illustration of this is given in Table II.

From what has been given before, it is apparent that the magnitude of the membrane potential is determined by the value of the Donnan ratio λ . When λ has the value 1, its logarithm will be zero and there will be no membrane potential. In protein systems this is true for the pH value that corresponds to the isoelectric point. When λ is greater than 1, the membrane potential will have a finite value, and when λ is less than 1, the membrane potential will also have a finite value but with the opposite sign. This, in protein systems, corresponds to changing the pH from the acid to the alkaline side of the isoelectric point and thus also changing the charge on the protein from a positive to a negative value. The effect of the pH of the solution and the valency of the acid used on the membrane potentials are shown in Fig. 7.

The curves in the figure show the influence, which is characteristic for protein solutions, of monovalent and divalent acids on the membrane potentials of 1 per cent gelatin solutions at varying pH values. The curve further shows that the membrane potential is zero at the isoelectric point (pH 4.8), reaches a maximum at about pH 4.0, and then drops off again toward zero as the amount of acid is increased.

The curve also shows that at the same pH value the divalent acids, sulfuric and sulfosalicylic, produce only two thirds of the membrane potential that is obtained with monovalent acids. This influence of valency follows from what has been shown in the development of equations (2) and (5); viz., that an increase in the valency of the diffusible ion results in an increase in the degree of the algebraic equation of the membrane distribution.

Thus, for a monovalent acid we have from equation (2)

$$X = \sqrt{Y(Y+Z)}$$
 and $\frac{X}{Y} = \frac{\sqrt{Y(Y+Z)}}{Y} = \sqrt{1 + \frac{Z}{Y}}$

Since $X/Y = \lambda$, the membrane potential becomes

$$E = \frac{RT}{F} \ln \sqrt{1 + \frac{Z}{Y}} = \frac{1}{2} \frac{RT}{F} \ln \left(1 + \frac{Z}{Y}\right)$$
 (26)

Now, for a bivalent acid, it follows from equation (5) that

$$X = \sqrt[3]{Y^2(Y+Z)}$$

then

$$\frac{X}{Y} = \frac{\sqrt[3]{Y^2(Y+Z)}}{Y} = \sqrt[3]{\frac{Y^2(Y+Z)}{Y^3}} = \sqrt[3]{1 + \frac{Z}{Y}}$$

From this

$$E = \frac{RT}{F} \ln \sqrt[3]{1 + \frac{Z}{Y}} = \frac{1}{3} \frac{RT}{F} \ln \left(1 + \frac{Z}{Y} \right)$$
 (27)

From the two equations, (26) and (27), the membrane potentials at equal values of Z and Y are seen to be in the ratio of 3 to 2 between a monovalent and a bivalent acid. This is exactly as has been found and is in good agreement with the data plotted in Fig. 7.

We come now to the explanation for the characteristic curves obtained for the membrane potential in protein systems with change of pH. The characteristic curves show a zero membrane potential at the isoelectric point. The membrane potential then increases as the pH is shifted to either the acid or alkaline side of the isoelectric point, passes through a maximum, and falls off again toward zero in highly acid or alkaline solutions. From equation (23) we see that the membrane potential is proportional to the logarithm of the Donnan ratio, λ . Accordingly, the change in the membrane potential should follow the change of λ as the pH is varied. Equations for this change have already been developed; namely, equation (11) for a non-diffusible weak base, equation (14) for a non-diffusible weak acid, and equation (21) for an amphoteric electrolyte.

In discussing equations (11) and (14) we have already seen that these equations only show an approach of the value of λ to 1 as either the acidity or alkalinity increases. The only difference between these two equations in this respect is that in equation (14) λ is less than 1, while in equation (11) λ is greater than 1, which means that the potential calculated by equation (14) is of the opposite sign to that calculated from equation (11). Both of the equations show that for either a weak base or a weak acid alone, the membrane potential can only numerically decrease as the hydrogen ion, represented by V, and the hydroxyl ion, represented by V, are respectively increased.

On the other hand, equation (21), representing the amphoteric dissociation of a protein, accounts very well, at least qualitatively, for the characteristic membrane potential curves.

From equation (21), the membrane potential equation becomes, on introduction of the numerical value of the constants,

$$E = 0.05915 \log \lambda = \frac{0.05915}{2} \log \left[1 + \frac{C(Y^2 - I^2)}{Y(Y^2 + YK_1 + I^2)} \right]$$
 (28)

From this it is readily seen that at the isoelectric point when $Y^2 = I^2$, λ becomes 1, the logarithm of which is zero and, accordingly, the membrane potential is zero. When Y is a hydrogen ion value greater than I, the value of λ will at first increase and the

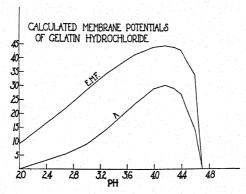


Fig. 8. The change of the Donnan ratio, λ , and of the membrane potentials of gelatin hydrochloride with pH. The data used in calculating the curves were taken from Hitchcock, D. I., J. Gen. Physiol., 9, 105 (1925). They are: I = pH 4.7, pK₁ = 3.62. Concentration of gelatin, C in terms of equivalence, = 0.01 N.

membrane potential will necessarily increase also. The membrane potential will pass through a maximum and fall off again as Y is increased more and more in comparison to I, for it has already been pointed out that for such a state, equation (21) reduces to equation (11) from which only a fall in λ and correspondingly a decrease in membrane potential are possible. A decrease in hydrogen ion to values less than I gives a value for λ less than 1. This also means that there will be a numerical increase in the membrane potential but opposite in sign to the membrane potential on the acid side of I. In this way, equation (22) is seen to give a representation of the curves obtained for the membrane potentials of protein systems. A plot made from equation (22) is shown in Fig. 8. A value for C of 0.01 N, and the values of the dissociation constants for gelatin taken from Hitchcock (5); namely, I = pH 4.7 which is a $[H^+]$ of 2.5×10^{-5} and $K_1 = 2.4 \times 10^{-4}$, were used in the calculations for the curve. The plotted figure corresponds very well in form with the experimental curve for gelatin given in Fig. 7, and the point of maximum membrane potential, 4.15, agrees fairly well with the pH 4.0 found by Loeb.

Neutral salts have experimentally been found to only depress the magnitude of the membrane potentials at a given pH value. The depressing action is determined by the valency of the ion of

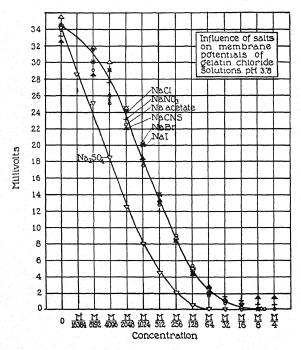


Fig. 9. Curves showing the depressing effect of neutral salts on the membrane potentials. The experiments were performed on gelatin hydrochloride solutions at pH 3.8. All salts with monovalent anions have within the limits of experimental accuracy the same depressing effect on the membrane potentials of gelatin chloride solutions at pH 3.8, while the depressing effect of Na₂SO₄ is much greater.

(Loeb, J., and Kunitz, M., J. Gen. Physiol., 5, 693 (1922-23); Loeb, J., Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 206.)

opposite charge to the charge on the protein. This is well brought out in Fig. 9, which shows the depression of the membrane potential of gelatin chloride solutions at pH 3.8 produced by monovalent and bivalent salts. This effect is readily seen to follow from equation (7). The ionization of the protein is not altered, while the concentration of diffusible ions becomes increased by a neutral salt.

The membrane potential should be expected to change with the

concentration of the protein, increasing as the protein is increased. The exact effect of the protein concentration on the membrane potential is shown by the term C of equation (21) in which it represents the equivalent concentration of the protein. This was verified experimentally by Loeb who has found that the membrane potential does increase as the protein concentration is increased.

6. OSMOTIC PRESSURE

The osmotic pressure of colloidal solutions—including proteins—is another important physical property which is influenced by the Donnan membrane equilibrium. The osmotic pressure has been one of the properties of protein solutions which has been extensively studied, particularly in connection with attempts to determine the molecular weights of proteins. However, before the development of the concept of the isoelectric points of proteins by Michaelis (10) and of the influence of the Donnan membrane equilibrium, the published results on the subject were in a state of hopeless confusion. Thus, as an illustration, Lillie (11) arrived at the conclusion that acids increase, while alkalies and neutral salts decrease the osmotic pressure of protein solutions. A correct understanding of the influence of electrolytes on the osmotic pressure of proteins came with the work of Sörensen (12) and particularly of Loeb (3).

The influence of the Donnan distribution on osmotic pressure may be approached by again considering the system represented by Fig. 1 when equilibrium has been reached. In the figure it is seen that there is a greater osmotic concentration on side (1), containing the protein chloride, than there is on side (2), containing only hydrogen chloride. From this it is to be concluded that the escaping tendency of the water on side (2) is greater than on side (1), and consequently there will be a diffusion of water from (2) to (1) until a hydrostatic pressure is established that equalizes the activity of the water on the two sides of the membrane.

For the sake of simplicity in developing the quantitative treatment, it will be assumed that the osmotic pressure follows van't Hoff's law,

 $P = RTC \tag{29}$

in which P represents the osmotic pressure, R the gas law constant, T the absolute temperature, and C the molar concentration. Actually, van't Hoff's law of osmotic pressure holds for only very

dilute protein solutions, as has been pointed out by Adair (13) and by Burk and Greenberg (14). A rigorous thermodynamic treatment of the osmotic pressure of Donnan systems is given by Adair.

From Fig. 1 it is seen that the terms determining the osmotic pressure on side (1) are: the $[H^+]=Y$, and $[Cl^-]=Y+Z$, and the molar protein concentration, which will be represented by a. These terms alone will not completely determine the osmotic pressure of the system, for there will be a counteracting osmotic pressure on side (2) due to the hydrogen chloride present which is determined in magnitude by the term 2X.

Accordingly, the total osmotic pressure of the system will be P = RT(2Y + Z - 2X + a) = RTe + RTa (30)

The term e, which is equivalent to 2Y+Z-2X, is often designated the Donnan osmotic pressure term, for it alone is affected by the Donnan equilibrium, while a is a function of the molecular weight of the protein only.

Since the molecular size of proteins and other colloids is usually very great, the osmotic effect due to the protein is often small and may be neglected in comparison to the Donnan osmotic pressure.

If the osmotic pressure is measured in terms of the height of a column of water at the temperature of 0°, equation (30) becomes

$$P = 2.315 \times 10^5 (e+a)$$
 millimeters of water (31)

In protein systems, the Donnan osmotic pressure, like the membrane potential, is largely determined by the pH of the solution and gives a curve of the same general form as the membrane potentials; i.e., zero at the isoelectric point, increasing and reaching a maximum as the pH is shifted to either the acid or the alkaline side of the isoelectric point, and then falling off again with still more increase of acid or alkali. Also, as would be expected, the valency of the diffusible electrolytes is a large factor in determining the magnitude of the osmotic pressure. Curves taken from Loeb showing that the osmotic pressure of 1 per cent gelatin solutions is a function of the pH and of the valency of the diffusible electrolytes are given in Fig. 10. The agreement between the observed and calculated values of the osmotic pressure on the basis of the simple Donnan theory is shown in Fig. 11.

From Fig. 10, the osmotic pressure in hydrochloric acid solution is seen to be about twice as great as in sulfuric acid solution at the same pH. The reason for the influence of the valence of the acid

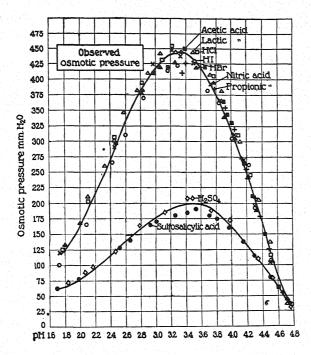


Fig. 10. Proof of valency rule for the influence of acids on the osmotic pressure of gelatin solutions. Influence of acids on the osmotic pressure of gelatin solutions. At the same pH the monovalent acids produce about double the osmotic pressure caused by the divalent acids. The influence of seven monobasic acids on the osmotic pressure of gelatin solutions is the same and about twice as high as that of the two dibasic acids.

(Loeb, J., and Kunitz, M., J. Gen. Physiol., 5, 665 (1922-23); Loeb, J., Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 113.)

can be understood from the following considerations. The Donnan osmotic pressure term for a protein in a solution of monovalent acid, as we have already seen, is given by the equation,

$$P = RT(2Y + Z - 2X)$$

For a bivalent acid, from the conditions for equilibrium shown in Fig. 2, the terms determining osmotic pressure on side (1) are $[H^+] = Y$ and $[SO_4^-] = (Y+Z)/2$. The counteracting osmotic terms on side (2) are $[H^+] = X$ and $[SO_4^-] = X/2$. From this it follows that the osmotic pressure is determined by

$$P = RT\left(\frac{3Y + Z - 3X}{2}\right)$$

Because the term, 3Y-3X, does not differ very greatly in magnitude from the term, 2Y-2X, with a monovalent acid, it is seen that the $\frac{1}{2}$ term in the equation for a divalent acid determines that the osmotic pressure will be about half of the value found in the case of a monovalent acid.

The form of the osmotic pressure curves obtained with the variation of the pH of the solution can be explained, as was the mem-

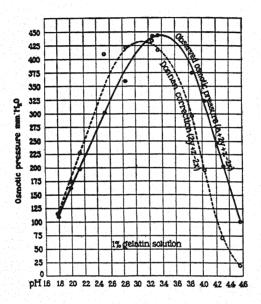


Fig. 11. Comparison of the calculated with the observed values of the osmotic pressures of gelatin hydrochloride solutions.

(Loeb, J., J. Gen. Physiol., 3, 691 (1920-21); Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 220.)

brane potential, by the equations for the membrane equilibrium in terms of the dissociation of the protein as a function of the pH.

It has been shown in equation (2) that

$$\lambda = \frac{X}{Y} = \frac{Y + Z}{X}$$

Substituting values of λ for equivalent terms in e=2Y+Z-2X, there is obtained

$$e = Y + \lambda X - 2X = Y + \lambda^2 Y - 2\lambda Y$$

This reduces to

$$e = Y(\lambda - 1)^2 \tag{32}$$

The same equation for e, the osmotic determining term, can be obtained from the more complex membrane distribution for an amphoteric colloidal electrolyte shown in Fig. 5. Substituting the values for λ , given by equations (11), (14), and (21), it is possible to calculate the osmotic pressure in terms of the hydrogen ion concentration and the corresponding ionization constants. We have already seen that, at the isoelectric point, λ becomes 1. On sub-

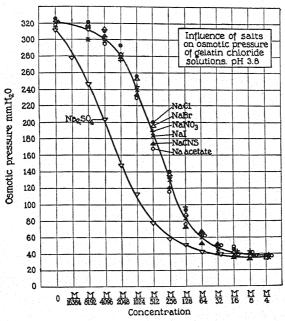


Fig. 12. Depressing effect of neutral salts on the osmotic pressure of gelatin hydrochloride solutions of pH 3.8. All salts with monovalent anions depress the osmotic pressure of gelatin chloride solutions of pH 3.8 to the same extent (within the limits of experimental accuracy). Na₂SO₄ depresses considerably more.

(Loeb, J., and Kunitz, M., J. Gen. Physiol., 5, 693 (1922-23); Loeb, J., Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 144.)

stituting this value in equation (32) we see that e, and correspondingly the osmotic pressure, is zero. It has been seen that in accordance with equation (21) the value of the Donnan ratio, λ , starting at the isoelectric point, first increases as Y, the hydrogen ion concentration of the protein solution inside the membrane, is increased, and then passes through a maximum. Since, from equation (32), the Donnan osmotic pressure is also a function of λ and Y, it is apparent that the osmotic pressure will follow a parallel course with change of acidity.

It is of interest to note that, on substituting equation (11) or (14) for λ in equation (32), it is found that the osmotic pressure for a non-diffusible base or acid, unlike the membrane potential, will also rise and pass through a maximum with change of the pH of the solution. By the use of the differential calculus, Hitchcock (5) has shown that the maximum for the osmotic pressure on the acid side of the isoelectric point is given by the equation, $Y = \sqrt{K_1 X}$, and on the alkaline side of the isoelectric point by $Y = \sqrt{k_a X}$.

The addition of neutral salts decreases the osmotic pressure except when the protein is at the isoelectric point. This fact was pointed out by Lillie (11). The amount of the depressing action by a salt is a function of the valence of the ion of opposite charge to that of the protein. The reason for the depressing effect on osmotic pressure is explained in the same way as its effect on the membrane potential. Adding a neutral salt does not change the ionization of the protein and, accordingly, the Z term remains unchanged, while both Y and X are increased. But, from equation (2), when Y and X are increased in comparison to Z, the values tend to approach each other so that the value of e is diminished.

The depressing effect of neutral salts on the osmotic pressure of a gelatin chloride solution at pH 3.8 is shown graphically in Fig. 12.

7. SWELLING

The imbibition of water and the swelling of protein gels under various experimental conditions is a subject that has received an enormous amount of attention from many investigators. The two proteins that are the prototypes of protein gels are fibrin, which forms only an irreversible gel, and gelatin, which gives a readily reversible gel. Of all the gelling substances, gelatin has been by far the most studied. In this section the results that have been obtained for gelatin will be discussed. There seems to be no reason to doubt that the deductions which have been made from the study of gelatin can be generally applied to other protein gels.

The swelling of gelatin under all conditions cannot be explained by a single theory. This swelling may be divided into three types: (a) swelling of dry gelatin in small amounts of liquid, (b) swelling of dilute isoelectric gelatin, and (c) the swelling in dilute acid or alkali, including the effect of neutral electrolytes on this type of swelling. The first two types of swelling are not to be explained by the Donnan theory and accordingly will not be considered in this section.

The explanation of the swelling of gelatin in dilute acid or alkali, and the influence of neutral salts thereon, were shown by the work of Proctor and Wilson (15) and later by that of Loeb (3) to be dependent on the Donnan theory of membrane equilibrium.

According to the theory of this type of swelling, when an acid such as HCl is added to gelatin, the gelatin combines with some of the acid, forming an ionized salt like gelatin chloride. In the gel, the gelatin ion cannot diffuse away from its fixed position, while, on the contrary, the ions of free acid are freely diffusible in the gel. As a result of this, a membrane equilibrium is set up between the interior of the gel and the outer solution no different in kind from the membrane equilibria we have already considered. And just as with the systems we have been considering, there is a greater concentration of osmotically active ions in the gel than in the external liquid. This excess of osmotic pressure leads to a diffusion of water into the gel which gives rise to the swelling. If there were no counteracting force to oppose the osmotic force, the swelling would continue indefinitely. However, according to the theory, the cohesive forces that tend to hold the gel together supply the mechanism which limits the amount of the swelling.

Proctor and Wilson (15), in developing a quantitative theory for the swelling of gelatin in dilute acids, made use of the following assumptions:

- 1. Gelatin combines with the acid as a weak monovalent base whose ionization obeys the mass law. The development of this conception on the membrane equilibrium distribution has already been considered and the results are embodied in equation (10), $Z = CY/(K_1+Y)$, and in equation (11), $\lambda = \sqrt{1+[C/(K_1+Y)]}$.
- 2. The gel itself, because of its fixed structure, constitutes a membrane impervious to ionized gelatin, but readily permeable to hydrogen ion and the anion of the diffusible acid.
- 3. The swelling is due to the osmotic effect of the excess of diffusible ion resulting from the Donnan distribution. This, as has been shown, is given by the equation,

$$e = 2Y + Z - 2X$$

4. The cohesive forces of the gel which resist the swelling are elastic and obey Hooke's law. This is embodied in the equation,

where C is a constant corresponding to the bulk modulus of elasticity of the protein, and V is the increase in volume, measured in cubic centimeters of the protein. The term V is also equivalent to the reciprocal of the concentration of the protein in the gel, provided the initial volume before swelling may be considered negligible.

From the assumptions and fundamental equations, there can be developed an equation for swelling in terms of V, Y, and the constants, C and K_1 , in the following way:

Since the dilution is the reciprocal of the concentration, the term C for the protein concentration in equation (10) can be written as C=1/V and equation (10) becomes $Z=Y/V(K_1+Y)$. From equation (2), $X=\sqrt{Y(Y+Z)}$ and e=2Y+Z-2X. On substituting there is obtained $X=Y+\sqrt{eY}$ from which $e=Z-2\sqrt{eY}$. Now, substituting e=CV from equation (33), and rearranging, there is obtained

$$V(K_1 + Y)(CV + 2\sqrt{CVY}) - Y = 0$$
(34)

If the values of the constants K_1 and C are known, then, in turn, Y, e, Z, and X can be evaluated. However, in actual practice, K_1 and C have to be evaluated from the experimental data. In the experiments of Proctor and Wilson this was done in the following way. Sheets of thin, purified gelatin were cut into discs of 1 gram each. The gram discs were put into a series of stoppered bottles containing 100 cc. of known acid concentration. After an interval of 48 hours to allow attainment of equilibrium, the solution which remained was drained off and titrated with standard alkali. The swollen gelatin discs were quickly weighed and the volume of the swelling, V, was calculated from the increase in weight. The swollen gelatin was then put back into the same bottles and enough dry sodium chloride was added to saturate the solution. This caused the gelatin to contract and give up the absorbed solution. After another 24 hours, when the imbibed solution had been expelled by the salt, it was drained off and titrated to determine the amount of free acid which had been absorbed by the gelatin. A small amount of about 1 cc. always remained unexpelled by the salt, and a correction for this was made. The acid unaccounted for by these two titrations was assumed to have combined with the gelatin.

From the data obtained in the experiments, it was possible to

evaluate the constants and test the applicability of equation (34). The constant, K_1 , in Proctor and Wilson's experiments was found to have the value, 1.5×10^{-4} . The values for the constant C were found to depend on the temperature and the quality of the gelatin. A curve that illustrates Proctor and Wilson's results is given in Fig. 13.

Loeb extended the range of Proctor and Wilson's experiments by showing that the same correlation existed between swelling and

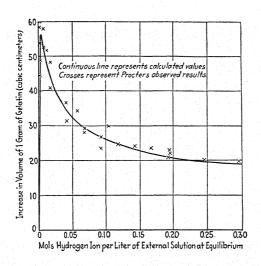


Fig. 13. Observed and calculated values for the degree of swelling of gelatin as a function of the concentration of hydrochloric acid in the external solution at equilibrium.

(Proctor, H. R., J. Chem. Soc., 105, 313 (1914); Bogue, R. H., Colloidal Behavior, New York and London, 1924, Vol. I, p. 18.)

the membrane potential as between the osmotic pressure and the membrane potential. The effect of neutral salts in diminishing the amount of swelling was also shown by Loeb to fit in well with the Donnan theory. In Loeb's experiments, samples of powdered particles of isoelectric gelatin were put into a given volume of the desired solution and allowed to remain a number of hours to bring about an equilibrium between the gelatin particles and the outer solution. The suspended particles were then separated from the solution by filtration and the amount of the swelling was determined. The swollen particles were then melted in order to measure the pH of the gelatin solution and the outer solution. From pH measurements the membrane potential was calculated, or, by a

modification of the method shown in Fig. 6, the membrane potential was determined directly. Illustrations of Loeb's experi-

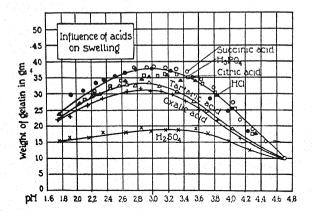


Fig. 14. The influence of acids of varying valency on the swelling of gelatin. (Loeb, J., and Kunitz, M., J. Gen. Physiol., 5, 665 (1922-23); Loeb, J., Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 122.)

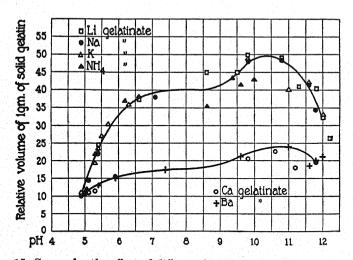


Fig. 15. Curves for the effect of different bases on swelling. Those for LiOH, NaOH, KOH, and NH₄OH are practically identical and about twice as high as those for Ca(OH)₂ and Ba(OH)₂.

(Loeb, J., J. Gen. Physiol., 3, 247 (1920-21); Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 124.)

mental results are plotted in Figs. 14 and 15. The figures show the effect of the pH and the valency of the acids and bases on the swelling on the acid and alkaline side of the isoelectric point.

8. HETEROGENEOUS EQUILIBRIA, VISCOSITY AND ULTRAFILTRATION

In the beginning of this chapter it was pointed out that the important condition for the establishment of a Donnan equilibrium is that one or more ionic species are restrained from diffusing throughout the whole system, while the other ions present are able to do so. This condition can be produced, as it has already been seen, by the non-diffusible ion forming part of a fixed structure such as occurs in a gel. From the condition represented by a macroscopic, gross gel, it is no great step to extend the theory to apply to the ionic equilibrium between a submicroscopic colloidal micelle and the dispersion medium of the colloidal solution as has been done by the Wilsons (16, 17) and by Oakley (18). To do this, it need only be imagined that the colloidal macroscopic gel is subdivided into smaller and smaller units until the particles are of submicroscopic size. Even in their finest state of subdivision, the colloid particles are still very large in comparison to the solvent molecules and the diffusible ions of the system. Logically, on this consideration, it would be expected that the same formulation which governs the distribution of electrolytes between the inside of the gel and the solution which bathes the gel would also apply to the distribution between the colloidal micelle and the intermicellar liquid. The same quantitative equations that have been developed for the macroscopic state, it would be expected, should apply equally well to the system in the submicroscopic state.

This idea has been used by Wilson and by Oakley as a basis for explaining the stability of lyophobic sols and their flocculation by electrolytes. Using the illustration of a colloidal gold solution, Wilson (17, p. 21) points out that if there is a distribution of the Donnan type between the colloidal micelle and the dispersion medium, there would necessarily be a potential difference between the colloid and dispersion medium. This potential difference between the enveloping film of the colloid and the surrounding solution would serve to maintain the stability of the colloid. Furthermore, it will be at a maximum when there is no free salt present and will decrease and approach zero as more and more electrolyte is added to the solution. When enough salt has been added so that the potential difference is reduced to a value where the attractive forces between the colloidal particles are greater, then the particles will coalesce and the colloid will flocculate from the solution. Ac-

cording to Wilson, "It is at this point that the actual charges themselves come into play and probably determine the nature of the precipitate." The lowering of the potential difference between the colloid and the dispersion medium, it is to be noted, does not necessarily involve any decrease in the value of the electrical charge on the colloid particle itself.

(1) Ultrafiltration

It has been shown by Greenberg and Greenberg (19) that an important application following from the extension of the Donnan theory to the equilibrium between micelle and the intermicellar liquid is found in ultrafiltration. It has been the widely current view that the ultrafiltration of electrolytes from colloidal solutions is a simple filtration of the diffusible electrolytes in the same concentration in which they are present in the free solvent of the colloidal solution. This simple view, it has been shown, is not correct. Instead, there is found a very definite difference in concentration between the electrolytes in the ultrafiltrate as compared to the concentration in the original colloidal solution and this difference in distribution is quite analogous to that which would follow from the Donnan membrane theory.

The explanation that seems most plausibly to account for this effect is that in such an ultrafiltration, where the colloid is completely held back by the membrane, the ultrafiltrate is a measure of the composition of the intermicellar liquid. On the basis of what has been said before, it would follow that this type of ultrafiltration is an experimental demonstration of the Donnan distribution of the electrolytes between the intermicellar liquid and the colloidal micelles.

To give a concrete illustration of the subject, we may consider the results obtained for the ultrafiltration of sodium caseinate solutions containing sodium chloride and sodium sulfate, respectively. If there is ultrafiltered a small amount of liquid from a large amount of a sodium caseinate solution, no appreciable change will result in the composition of the colloidal solution. Then the concentration of sodium chloride in the ultrafiltrate will be given by the equation,

$$X = \sqrt{Y_0(Y_0 + Z_0)} \tag{35}$$

which is identical with equation (2), but in which the letters now have the significance that X is the concentration of NaCl in the

TABLE III			
Sodium Caseinate+	Na	Cl	,

Casein (1)	added		$\begin{bmatrix} \text{NaCl in} \\ \text{filtrate} \\ \text{(4)} \end{bmatrix}$ $\begin{bmatrix} \text{NaCl in} \\ \text{filtrate} \end{bmatrix}$ $\begin{bmatrix} -C_1 \\ C_2 \end{bmatrix}$		$\frac{F_{\text{NaCl}}}{C_{\text{NaCl}}}$ (6)	F _{NaCl} O _{NaCl} (7)
per cent	N	N	N	N		
1.95	0.0131	0.0062	0.0104	0.0109	0.95	1.68
1.95	0.0131	0.0100	0.0149	0.0151	0.99	1.53
1.30	0.0088	0.0114	0.0152	0.0151	1.00	1.34
1.75	0.0115	0.0148	0.0191	0.0197	0.97	1.32
1.75	0.0115	0.0246	0.0302	0.0300	1.00	1.23
2.82	0.0197	0.0443	0.0520	0.0532	0.98	1.18

The heading of column 6 in the tables, $F_{\rm salt}/C_{\rm salt}$, represents the ratio of salt experimentally found in the filtrate to the amount calculated. The column heading 7, $F_{\rm salt}/O_{\rm salt}$, gives the ratio of the salt concentration in the filtrate to the salt concentration in the original protein solution.

(Greenberg, D. M., and Greenberg, M., J. Biol. Chem., 94, 373, (1931).)

ultrafiltrate, Z_0 is the equivalent concentration of sodium in combination with the casein, and Y_0 is the concentration of sodium chloride as originally added to the caseinate solution.

If sodium sulfate is the salt added, then the concentration of sodium sulfate in the ultrafiltrate liquid is given by the equation,

$$X = \sqrt[3]{Y_0(Y_0 + Z_0)^2},\tag{36}$$

where X and Y_0 now represent sodium sulfate concentration in

Table IV Sodium Caseinate $+Na_2SO_4$

Casein (1)	NaOH added (2)	Na ₂ SO ₄ added (3)	Na ₂ SO ₄ in filtrate (4)	Calculated Na ₂ SO ₄ in filtrate (5)	$\frac{F_{\text{Na}_2\text{SO}_4}}{C_{\text{Na}_2\text{SO}_4}}$ (6)	$\frac{F_{\text{Na}_2\text{SO}_4}}{O_{\text{Na}_2\text{SO}_4}}$ (7)
per cent 2.78 1.68 1.97	N 0.0196 0.0115 0.0131	N 0.0121 0.0186 0.0242	N 0.0207 0.0250 0.0304	N 0.0230 0.0256 0.0323	0.90 0.98 0.94	1.71 1.34 1.24

The heading of column 6 in the tables, $F_{\rm salt}/C_{\rm salt}$, represents the ratio of salt experimentally found in the filtrate to the amount calculated. The column heading 7, $F_{\rm salt}/O_{\rm salt}$, gives the ratio of the salt concentration in the filtrate to the salt concentration in the original protein solution.

(Greenberg, D. M., and Greenberg, M., J. Biol. Chem., 94, 373, (1931).)

ultrafiltrate and caseinate solution, respectively. The agreement obtained between the experimentally determined values and the values calculated from equations (35) and (36) is shown in Tables III and IV. The apparatus in which the ultrafiltration experiments were carried out is shown in Fig. 16.

The last column in the tables, which has the heading, $F_{\rm salt}/O_{\rm salt}$, gives the ratio of the concentration of salt in the ultrafiltrate to the concentration of the salt as originally added to the case in at solu-

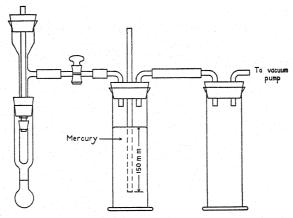


Fig. 16. Ultrafiltration apparatus.

(Greenberg, D. M., and Gunther, L., J. Biol. Chem., 85, 491 (1930).)

tion. The good agreement between the experimental values and the figures calculated from equations (35) and (36), as is shown in the tables, strongly favors the theory of a Donnan effect in micellar equilibrium.

(2) Viscosity

The hypothesis of the existence of a Donnan distribution between the colloidal micelle and the intermicellar liquid has been used by Loeb as the basis of a theory for explaining the influence of electrolytes on the viscosity of protein solutions. According to Loeb (3), there are two kinds of viscosity, one which is common to all solutions both, crystalloidal and colloidal, but which has no connection with colloidal behavior, and a second type found only in colloidal solutions which depend upon the swelling of ultramicroscopic colloidal particles in the solution. This second type of viscosity, which is specific for colloidal phenomena, depends, according to Loeb, upon the Donnan equilibrium.

In Fig. 17 there is shown a curve of the influence of the pH and

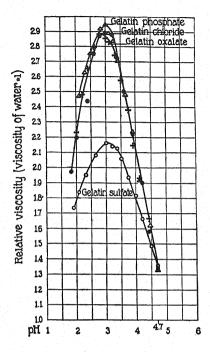


Fig. 17. Curves representing relative viscosity of 0.8 per cent solution of originally isoelectric gelatin brought to different pH. The curves for relative viscosity of gelatin chloride, phosphate, and oxalate are practically identical. Relative viscosity is given as time of outflow of gelatin solution divided by time of outflow of water through viscometer at 24°.

(Loeb, J., J. Gen. Physiol., 3, 85 (1920-21); Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 127.)

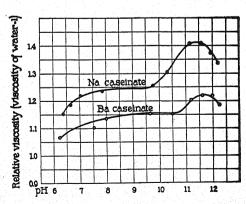


Fig. 18. The influence of bases on the relative viscosity of casein solutions with varying pH.

(Loeb, J., J. Gen. Physiol., 3, 547 (1920-21); Proteins and the Theory of Colloidal Behavior, 2nd edition, New York and London, 1924, p. 131.)

the valency of the ions of certain acids on the relative viscosity of gelatin, and in Fig. 18 is shown the influence of bases on the viscosity of casein solutions.

As will be seen, there is a close resemblance between the shape of the curves for the effect of pH on the viscosity and the curves that have been obtained for the effect of the pH on osmotic pressure and the swelling of proteins. The explanation offered for the similarity in the curves of these properties of protein solutions by Loeb is that ". . . although protein solutions may be and primarily are true solutions consisting of isolated protein ions and molecules distributed equally through the water, they contain under certain conditions submicroscopic solid particles of protein. The viscosity of protein solutions is only influenced in the same way by electrolytes as is the osmotic pressure when such solid protein particles are present in considerable numbers and when they are capable of swelling."

9. BIOLOGICAL APPLICATIONS OF THE THEORY OF MEMBRANE EQUILIBRIA

Jacques Loeb concludes his book on *Proteins and the Theory of Colloidal Behavior* with the statement: "If Donnan's theory of membrane equilibria furnishes the mathematical and quantitative basis for a theory of colloidal behavior of the proteins, . . . it may be predicted that this theory will become one of the foundations on which modern physiology will have to rest." Partially, at least, Loeb's prediction has been fulfilled. The Donnan theory has furnished the key for the explanation of the mechanism of the exchange and of the distribution of the electrolytes in the blood between the blood corpuscles and the plasma and of the distribution of electrolytes between the blood stream and many of the extracellular fluids of the body. A great deal of the credit for the development in this direction is due to D. D. Van Slyke and his co-workers (20, 21).

For a long time it has been known that in normal blood the chloride and bicarbonate concentrations of the red cells are about half the values found in the plasma, and this inequality persists even though chloride, bicarbonate, and other anions are readily capable of diffusing in and out of the cells under the influence of changing CO₂ tension. On the other hand, the inorganic cations such as sodium and potassium are unable to diffuse from or into the red cells. It has also been shown that the hemoglobin of the

red cells is in combination with a large fraction of the readily exchangeable base of the blood (22), and that the osmotic pressure of the fluid within the cells is about equal to that of the plasma surrounding the cells. The only cation in the blood stream to which the red cells are readily permeable is hydrogen ion. Since only hydrogen ion and the crystalloidal anions of the blood are completely diffusible, while the metallic cations, hemoglobin, and the other protein ions are restrained from diffusing through the cellular membrane, we have the condition necessary for a Donnan membrane equilibrium. For the blood stream this can be written as:

$$\lambda = \frac{[H^{+}]_{s}}{[H^{+}]_{c}} = \frac{[Cl^{-}]_{c}}{[Cl^{-}]_{s}} = \frac{[HCO_{3}^{-}]_{c}}{[HCO_{3}^{-}]_{s}} = \frac{[A^{-}]_{c}}{[A^{-}]_{s}}$$
(37)

in which λ is the Donnan distribution ratio. Since in the blood stream the proteins are on the alkaline side of their isoelectric point, λ will have a value of less than 1. Van Slyke (20, p. 22) showed that from equation (37) there could be derived the equation for λ , given below; namely,

$$\lambda = 1 - \frac{[Hb^{-}]}{2[B^{+}]_{c} - [Hb^{-}]}$$
 (38)

in which [Hb⁻] represents the equivalent concentration of the hemoglobin and [B⁺], the concentration of the base in the corpuscles. From this equation it is seen that the distribution of the diffusible ions between the cells and the plasma is largely determined by the base bound to the hemoglobin.

The base-binding power of hemoglobin and the Donnan theory offer a rational explanation for the following properties of the blood: (a) the cells contain more base in proportion to water than does the plasma, but contain much less Cl- and HCO₃-; (b) the cell content is more acid than is the plasma; (c) the cells carry most of the buffer alkali (as the salt of hemoglobin) of the blood which is available to combine with carbonic or other acids entering the blood stream; (d) the cells absorb water from the serum when CO₂ or other acids enter the blood; and (e) at the same time, Cl- passes from the plasma to the cells.

The distribution of electrolytes between the blood plasma and the body fluids outside of the circulation, such as lymph, synovial fluid, and various transudates, is also explainable by the membrane equilibrium theory. The walls of the capillary blood vessels are impermeable to the plasma proteins, but are permeable to the crystalloidal electrolytes and water of the blood. From the analyses of plasma and intercellular body fluids, it appears that there exists a Donnan equilibrium between the blood and these fluids, with an inequality of cation and anion distribution. The distribution found by analysis is quantitatively in agreement with that calculated from the amount of alkali neutralized by the plasma proteins (23, 24).

10. ANOMALOUS OSMOSIS

From thermodynamic considerations, it is readily seen that when a solution is separated from its solvent by a semi-permeable membrane, and the two liquids are under the same pressure, there should be a flow of liquid from the solvent (or more dilute solution) to the more concentrated solution. Moreover, if the solution obeys the perfect solution laws, the rate of osmotic flow from dilute to concentrated solution should be proportional to the concentration difference between the two.

However, experimental studies have brought to light many systems that do not obey the ideal conditions pictured by thermodynamic theory. The deviations that are found may be in the direction of a greater, a less, or even a completely reversed flow from that expected theoretically. When there is a reversed flow; i.e., the liquid flows through the membrane from the concentrated to the dilute solution, the phenomenon is termed negative osmosis. When the flow obtained is qualitatively in the right direction, but is either greater or less than directly proportional to the concentration difference on the two sides of the membrane, then the phenomenon is termed anomalous osmosis. As we shall see, there is no essential difference between anomalous and negative osmosis. The effect obtained depends upon the magnitude and sign of the electrical forces concerned.

In biological systems, anomalous and negative osmosis are frequently encountered. For example, the formation of urine by the kidneys is apparently an example of negative osmosis.

While anomalous osmosis is a kinetic phenomenon far removed from the equilibrium state, it will be convenient to treat the subject in this chapter because of its relation to membranes and membrane potentials. The general aspects of the subject have been thoroughly studied by Girard (25), Freundlich (26), Bartell and his co-workers (27, 28), and by Loeb (29). Loeb especially determined the effect of proteins and the valency of electrolytes on osmosis.

Experimentally, anomalous osmotic effects have been obtained with a great variety of membranes; e.g., collodion, porcelain, pro-

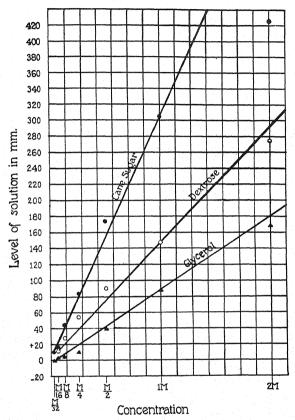


Fig. 19. Osmosis of non-electrolytes as a function of concentration. Within concentrations of M/32 and 1 M the initial rise of liquid in 20 minutes is in direct proportion to the concentration, as van't Hoff's law demands.

(Loeb, J., J. Gen. Physiol., 2, 176 (1919-20).)

tein, and such animal and vegetable membranes as gold beater's skin and parchment paper.

The cause of anomalous osmosis is to be attributed primarily to the operation of electrical forces due to the electrical charge of the membrane, the nature of the electrolytes in the solution, and the differences in their concentration on the two sides of the membrane.

With Bartell (28) it may be stated that in all osmotic systems, there will be a tendency for a normal osmotic flow, but there may be superimposed upon the normal osmosis an osmosis due to electrical effects which may work in the same direction as the normal flow, giving an abnormally high positive osmosis, or the effect may

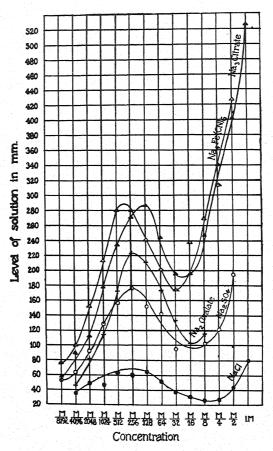


Fig. 20. The effect of valence and electrolyte concentration on the anomalous osmosis observed with a collodion membrane.

(Loeb, J., J. Gen. Physiol., 2, 177 (1919-20).)

work in the opposite direction, which, depending upon the magnitude, will give a low or even negative osmosis.

To illustrate the principle involved in the statement above, we may consider some of the experimental results obtained by Loeb. In Fig. 19 are shown the pressures resulting from the osmotic flow of certain non-electrolyte solutions through a collodion membrane. The osmosis obtained with these is linear and is proportional to the concentration, which is what would be expected in the ideal

case. However, when the osmosis is carried out with electrolytes the results obtained are quite different, as is shown by the curves plotted in Fig. 20. For osmosis through untreated collodion membranes, Loeb arrived at the conclusion that water diffuses into a solution of an electrolyte as if the water were positively charged

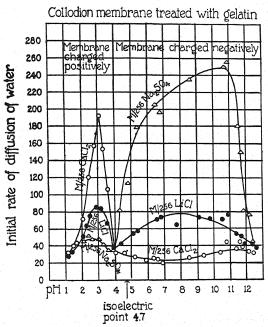


Fig. 21. The influence of the sign of the electrical charge of a membrane on anomalous osmosis. Collodion membranes previously treated with 1 per cent gelatin solution. Abscissae, pH (logarithms of hydrogen ion concentration with minus sign omitted). Ordinates, initial rate of diffusion of water from pure water through collodion membranes into solutions of salt indicated. Reversal of sign of charge of membrane at pH = 4.0; i.e., on the acid side of the isoelectric point of gelatin.

(Loeb, J., J. Gen. Physiol., 2, 577 (1919-20).)

and as if it were attracted by the anions and repelled by the cations of the electrolyte in solution, with a force which increases with the valency of the electrolyte. Hydrogen ion has but little effect on the osmosis through pure collodion membranes. However, when protein coated collodion membranes are employed, the relations obtained become more complicated and are very strongly influenced by the pH of the solution.

Untreated collodion membranes always remain negatively charged even though the electrolyte used is an acid. With protein coated membranes, on the contrary, the sign of the charge of the membrane, and consequently the osmosis, is very much dependent on the pH of the solution. As is shown in Fig. 21, the diffusion of the water in the protein coated membranes is found to alter with change of pH as if the membrane changed in sign from positive on the acid side of the isoelectric point of the protein to negative on the alkaline side. One of the most interesting effects found by Loeb for electrolytes is that higher valent cations, such as cerium or aluminium, are able to change the charge of protein coated membranes to positive even on the alkaline side of the isoelectric

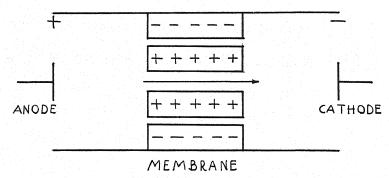


Fig. 22. Diagram representing the flow of water through a single capillary of a membrane. The electrical orientation is indicated. The flow takes place under the influence of a direct current.

point of the protein. Presumably, this is accomplished by the formation of positively charged complex ions by the reaction of the high valent ions with the protein.

A theory that gives a satisfactory explanation of anomalous osmosis has been elaborated through the work of Girard (25), and more completely developed by Bartell (27). The clue for the foundation upon which this theory rests comes from a consideration of the related phenomena of electrical osmosis. To illustrate this, let us consider a cell containing a dilute solution of an electrolyte such as is indicated by Fig. 22. The two compartments of the cell are separated by a porous membrane. When an electric current is passed through such a cell, water will flow from one compartment to the other. The direction of the flow will be determined by the electrical charge of the porous membrane and the direction of the passage of the electrical current through the cell. In the cell pictured in Fig. 22, in which the membrane is indicated to have a negative charge, the water will behave as if it were positively charged and will flow towards the cathode as shown by the arrow.

Quantitatively, the amount of water transferred in such an experiment is given by the equation of Helmholtz,

$$V = \frac{qeED}{4\pi\eta l}$$

in which V represents the amount of liquid transported, q is the cross sectional area of the membrane, l is the distance between the electrodes, e is the potential of the double layer inside the pores of the membrane, E is the externally applied electromotive force, D the dielectric constant, and η is the viscosity of the solution. From the equation it follows that the transference of liquid through a given membrane is directly proportional to the two electrical potentials E and e of the cell.

The flow of water through a membrane resulting from the application of a direct current, as is indicated by Fig. 22, is an experimental fact, independent of any theories. However, the Helmholtz equation which accounts for the amount of water transferred, is based on the conception of an electrical double layer existing between the walls of the material in the capillaries of the membrane and the fluid film wetting the capillaries. According to this view, the capillary walls carry a charge, the sign of which is dependent on the nature of the materials composing the membrane. To balance this charge, from the principle of electroneutrality, there must be situated a charge of opposite sign in the fluid film of liquid in the capillaries. When an external electrical potential is applied, in accordance with the laws of electrochemistry, the charged components of the double layer tend to move towards the electrodes. However, the charge in the membrane surface is fixed and cannot migrate. The fluid, on the other hand, has no such restraint, so that the force produced by the electrical potential causes the charged fluid to move to the electrode of the charge opposite to its own.

If there is a similarity between anomalous osmosis and electrical osmosis, there must be an explanation to account for the source of the potentials analogous to E and e in order to supply the driving force for the osmosis. According to Bartell, the potential analogous to E is supplied by the diffusion potential produced by the diffusion of the electrolytes through the pores of the membrane and set up because of the unequal migration velocities of the ions passing through the membrane. Loeb agrees with this except that, in his opinion, the diffusion potentials are modified from the values that

would be obtained if no membrane were present, because of the influence of the membrane on the ionic migration. Work on the permeability of membranes such as that of Michaelis and Perlzweig (30) supports Loeb's views.

The potential comparable to e is due to the charge on the pore walls of the membrane. According to Freundlich (26) and Bartell (27) and others, this charge is produced by selective "adsorption" of cations or anions by the material of the membrane. The recent ideas on colloidal dissociation (see Pauli and Valkó (31)) explain the source of the membrane charge equally well. As Loeb has

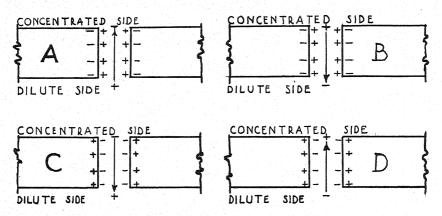


Fig. 23. Diagram showing the direction of the osmosis through a capillary of a membrane. The direction is determined by the electrical orientation of the membrane.

pointed out, the charge on protein coated membranes is due to the formation of ionized protein salts.

If the analogy between electrical osmosis and anomalous osmosis is accepted, then the course of the osmosis can be predicted from the electrical orientation of the system in the following way. In Fig. 23 is given a diagram which shows that four different conditions of electrical orientation may exist. It is seen that if the electrical orientation is as given by the diagrams A and D, there will be an increased flow from the dilute to the concentrated solution, which will give an abnormally positive osmosis; while, if the orientation is that indicated by B and C, the osmosis will be low or even negative. This theory of anomalous osmosis is in harmony with the experimental findings and is supported by the fact that a potential difference between the two sides of the membrane can

be shown to exist and to have an electrical charge that agrees with the electrical orientation postulated from the theory.

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CHAPTER XV

SOME THERMODYNAMICAL CONSIDERATIONS OF AMINO ACIDS, PEPTIDES, AND RELATED SUBSTANCES

By

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1. INTRODUCTION

Thermodynamics is primarily a treatment of the energy relations in physical and chemical systems. These energy changes fall into two categories. In one are quantities which we may designate as ΔU , the increase in total energy content; ΔH , the increase in heat content; q, the heat absorbed from the surroundings. The governing principle here is known as the law of the conservation of energy or as the first law of thermodynamics. Lewis and Randall's statement of this law is "Any system in a given condition contains a definite quantity of energy, and when this system undergoes a change, any gain or loss in its internal energy is equal to the loss or gain in the energy of the surrounding systems."

In the second category are quantities designated as ΔF , the increase in free energy; E, the potential difference in a galvanic cell; ΔS , the entropy change. These terms and concepts are developments of the second law of thermodynamics. It is difficult to give this law a statement which is rigorous and general and at the same time useful for application to a specific case. It is the second clause in Clausius' celebrated summary of the findings of thermodynamics "Die Energie der Welt ist konstant; die Entropy der Welt strebt einem Maximum zu." Lewis and Randall's statement is "Every system which is left to itself will, on the average, change toward a condition of maximum probability." Noyes and Sherrill expressed this law as follows: "A process whose final result is only a transformation of a quantity of heat into work is an impossibility." Ordinary macroscopic processes are implied in this statement.

The most useful deduction from this law for physiological chemical purposes is that in a given chemical reaction which proceeds spontaneously under isothermal conditions the difference between the free energy of the products, F_2 , and that of the reactants, F_1 , is always negative. In other words every spontaneous isothermal chemical reaction occurs in such a manner that $F_2 < F_1$.

The first law of thermodynamics has been applied to physiology since the days of Voit and Pettenkofer in the last half of the nineteenth century in the determination of the caloric food requirements of an individual and of the caloric content of foods. Another more recent use is as a guide to the discovery of the chemical reactions attending a given physiological process, for example muscular contraction. The heat produced in contraction and recovery is measured; the heat produced in the individual chemical reactions is estimated by a variety of methods. If all of the chemical reactions have been taken into account the sum of the heats of the chemical reactions is the same as that observed directly. We must be on guard here against a compensation of errors. A misleading pseudo balance is sometimes obtained although one or more reactions have been omitted from the balance sheet.

2. CONCEPTS AND EQUATIONS DERIVED FROM THE FIRST LAW OF THERMODYNAMICS

(1) The Total Energy Content. We shall now define some of the terms used in connection with the energy changes considered from the point of view of the first law of thermodynamics.

The energy content, U, of a system is fully determined by the state of the system. A change in the energy content, ΔU , i.e., $U_2 - U_1$, is determined solely by the initial and final states. It is independent of the process in the course of which the change in state has occurred. From the first law of thermodynamics

$$U_2 - U_1 = \Delta U = (+q) + (-w) \tag{1}$$

It is important at the outset to pay particular attention to the convention regarding signs. $(U_2 - U_1)$, $(H_2 - H_1)$, and $(S_2 - S_1)$ are ΔU , ΔH , and ΔS respectively. When the system absorbs heat, i.e., gains from the surroundings, the sign of q is positive; when it performs work, i.e., loses energy to the surroundings, the sign of w is negative.

(2) The Heat Content. A more convenient quantity than U is H, the heat content. It is defined by the equation

$$H = U + PV \tag{2}$$

where P and V are the pressure and volume.

In a reaction where the only work done is a change in the volume of the system at constant pressure

$$w = PV_2 - PV_1 \tag{3}$$

Substituting this value for w into equation (1) it becomes

$$U_2 - U_1 = (+q) - P(V_2 - V_1) \tag{4}$$

$$\therefore q = (U_2 + PV_2) - (U_1 + PV_1) \tag{5}$$

Most reactions are studied at atmospheric pressure, i.e., at constant pressure. One of the conveniences of the term ΔH is that it is equal to the quantity of heat absorbed by the system when the reaction occurs in a constant pressure calorimeter. From equations (2) and (5)

$$\Delta H = q \tag{6}$$

Equation (6) is valid only for a constant pressure process.

(3) Heat of Combustion. Heats of combustion are usually carried out in a bomb under a high pressure of oxygen, i.e., in a constant volume calorimeter. Here the quantity of heat absorbed is ΔU (the numerical quantity will have a minus sign, since heat is given off). This value is used to obtain the more useful value of the change in heat content, i.e., the heat of combustion at constant pressure. The relation between the two quantities can be derived as follows

$$\Delta H = \Delta U + P \Delta V \tag{7}$$

Assuming that the gases behave as perfect gases

$$P\Delta V = \Delta nRT \tag{8}$$

where Δn is the number of moles of gas produced in the reaction less the number consumed. Equation (7) then becomes for a pressure of one atmosphere

$$\Delta H = \Delta U + \Delta nRT \tag{9}$$

(4) Heat of Formation. From the value of its heat of combustion at constant pressure the heat of formation of a substance at constant pressure can be computed. The definition of the heat of formation and its relation to the experimental quantities can be derived from consideration of the following reactions carried out at constant temperature and pressure:

(1)
$$\frac{1}{2}$$
N₂ (gas)+ $\frac{5}{2}$ H₂O (liquid)+2CO₂ (gas) \rightarrow CH₂(NH₂)COOH (solid) + $\frac{9}{4}$ O₂ (gas); ΔH_1

- (2) 2C (graphite) +2O₂ (gas) \rightarrow 2CO₂ (gas); $\Delta H_2 = 2\Delta H_{(CO_2,gas)}$
- (3) $\frac{5}{2}$ H₂ (gas)+ $\frac{5}{4}$ O₂ (gas) $\rightarrow \frac{5}{2}$ H₂O (liquid); $\Delta H_3 = \frac{5}{2}\Delta H_{(H_2O,liquid)}$

Reaction (1) is the reverse of the combustion of glycine, reactions (2) and (3) represent the formation of CO₂ and H₂O respectively from the elements.

Adding these three reactions we obtain

$$\begin{array}{l} \frac{1}{2}\mathrm{N_2~(gas)} + 2\mathrm{C~(graphite)} + \frac{5}{2}\mathrm{H_2~(gas)} + \mathrm{O_2~(gas)} \longrightarrow \mathrm{CH_2(NH_2)COOH~(solid)} \\ \Sigma\Delta H = \Delta H_1 + 2\Delta H_{(\mathrm{CO}_2,\mathrm{gas})} + \frac{5}{2}\Delta H_{(\mathrm{H_2O,liquid})} \end{array}$$

 $\Sigma\Delta H=$ Heat of formation of solid glycine from the elements at constant temperature and pressure. The heat of formation of the elements in the standard state by definition is taken as zero. The formation of the compound, as written, is the reverse of the combustion. Therefore $\Delta H_1=-$ (heat of combustion). Since the heat of combustion by our convention has a negative sign, the numerical value of ΔH will be positive. Hence

$$\Delta H_{\text{(glycine,solid)}} = \text{Heat of combustion (+numerical quantity)}$$

 $+2\Delta H_{\text{(CO}_2,\text{gas})} + \frac{5}{2}\Delta H_{\text{(H}_2\text{O},\text{liquid)}}$

It is understood here that all quantities refer to processes carried out at the same constant pressure and temperature.

The change in heat content is used not only in the consideration of energy changes from the point of view of the first law of thermodynamics, but also in such second law considerations as the estimation of the free energy change at one temperature from that given at another. It is also used in conjunction with the third law of thermodynamics to obtain free energy values by exclusively thermal measurements.

3. CONCEPTS AND EQUATIONS DERIVED FROM THE SECOND LAW OF THERMODYNAMICS

(1) The Free Energy. From the first law of thermodynamics it is not possible to predict the direction which a given spontaneous reaction will follow. ΔH may be either negative, i.e., the reaction is exothermic as in a combustion, or it may be positive, i.e., the reaction is endothermic as when sodium chloride dissolves in water at room temperature. The free energy change for a given spontaneous isothermal reaction, on the other hand, is a measure of the spontaneous driving force. The direction is always such that ΔF is a negative quantity. In non-isothermal processes ΔS , the increase in entropy, is more useful than ΔF . Since most physiological processes are isothermal we shall be mainly concerned with ΔF .

(2) Free Energy Changes. The usefulness of ΔF is exemplified by its relation to such an experimental quantity as the equilibrium constant, K.

$$\Delta F^{\circ} = -RT \ln K \tag{10}$$

When the result is expressed in calories R is 1.9869 and T is the absolute temperature.

The relation of the increase in free energy at one temperature T_1 , to the increase in free energy at another temperature T_2 , is expressed by the van't Hoff equation

$$d\left(\frac{\Delta F^{\circ}}{T}\right) = -\frac{\Delta H}{T^{2}} dT \tag{11}$$

For a short temperature interval where ΔH may be taken as constant, the integrated form of equation (11) is

$$\frac{\Delta F_2}{T_2} - \frac{\Delta F_1}{T_1} = -\Delta H \left(\frac{T_2 - T_1}{T_1 T_2} \right) \tag{12}$$

The free energy of dilution of a substance is given by the equations

$$\Delta F = RT \ln N_2/N_1 \tag{13a}$$

$$=RT \ln P_2/P_1 \tag{13b}$$

when N and P are mole fraction and pressure respectively. It is understood that N_2 , N_1 , P_2 , P_1 , are quantities representing the thermodynamic activities.

In dilute solutions a negligible error is incurred in replacing N by C, the concentration, or by m, the mole ratio. Here it is common to represent the activity by the expression $C\gamma$ or $m\gamma$ where γ is the activity coefficient to be applied to convert the concentration or mole ratio to the thermodynamic activity.

If ΔF is a measure of the driving force of a reaction, then at equilibrium, i.e., when the reaction comes to a standstill, at constant temperature and pressure

$$dF = 0 \tag{14}$$

This is a thermodynamic definition of the equilibrium state.

From this relation it follows that the free energy of a solute in the saturated solution in contact with the solute in its solid phase is the same as that of the solid. In other words

$$\Delta F_{\text{(saturated solution)}T_1P} = \Delta F_{\text{(solid)}T_1P}^{\circ}$$

(3) Standard Reference Solution. From this relation and equation (13) and knowing ΔF of the solid we can obtain the free energy of formation in solution.

For this purpose it is convenient to employ a standard reference solution. The hypothetical standard solution which we shall employ is one in which all the partial molal quantities of solute and solvent are those of the solution at infinite dilution. A partial molal quantity will be defined below. In our standard solution the activity coefficients, designated as γ , of the solute and solvent are unity. The activity of the solute in any other solution with reference to this standard solution will then be $m\gamma$ or $N_2\gamma$ according to whether we wish to describe the concentration of the solute as moles per 1000 grams of water or as a mole fraction. The mole fraction is preferable.

(4) Free Energy of Solution. The increase in free energy in transferring 1 mole of solute from a very large quantity of the saturated solution to a very large quantity of the standard solution is from (13a)

$$\Delta F_{\text{(1M)}} - \Delta F_{\text{(saturated)}} = RT \ln \frac{1}{1 + 55.51} / \frac{S}{S + 55.51}$$
 (15)

where S is the number of moles of solute per 1000 grams of solvent, and where the saturated solution is a perfect one, i.e., Henry's Law is obeyed.

From equation (15)

$$\Delta F_{(1M,T)} = \Delta F_T^{\circ} - RT \ln \frac{S}{S + 55.51} / 0.0177$$
 (16)

where ΔF_{T}° is the free energy of formation of the solid at the temperature T.

If the substance is capable of dissociating in solution and we wish to have the free energy of the neutral form we must substitute for S in the numerator of equation (15) $S\alpha$ where α is the undissociated fraction.

(5) Free Energy and the Equilibrium Constant. Reactions are carried out as a rule with the reactants and products at specified or determined concentrations, which are different for each substance. This is especially the case in physiological reactions. We may wish to compute the free energy change in order to ascertain whether the reaction will go spontaneously, or if not, the minimum driving force which must be applied to it by means of another reaction.

Let us consider the reaction

$$aA(\text{at }C_A) + bB(\text{at }C_B) \rightarrow eE(\text{at }C_E) + fF(\text{at }C_F)$$

A, B, E and F are dissolved substances behaving as perfect solutes and C_A , C_B , C_E and C_F are their concentrations. In this reaction

$$\Delta F = -RT \ln K = -RT \ln \frac{C_E^e C_F^f}{C_A^a C_B^b}$$
 (17)

Equation (17) is true for any concentration of reactants and products.

(6) Free Energy of Ions. If we wish to obtain the free energy of formation of an ion from that of the parent undissociated monovalent acid or base we may consider the hypothetical solution where the neutral molecule and the ions derived from it are all at 1 molal activity. We need not concern ourselves with how such a solution is to be obtained. In this solution the free energy change in ionization is given by equation (17). Since the reactants and products are all at 1 molal activity the equation becomes

$$\Delta F_{\text{ion}}^{\circ} = -RT \ln K$$

where K is the ionization constant. For polyvalent acids or bases

$$\Delta F^{\circ}_{n^{\text{th}}_{\text{ion}}} = -RT \ln K_1 K_2 \cdot \cdot \cdot K_n \tag{18}$$

according to the number of ionizable groups for the ion whose valency is n.

From equation (18) the free energy of the ion at 1 molal activity then is

$$\Delta F^{\circ}_{\text{ion},1M,T} = \Delta F^{\circ}_{\text{(neutral molecule},1M,T)} - RT \ln K_1 K_2 \cdot \cdot \cdot K_n$$
 (19)

The free energy of the hydrogen ion is taken as zero at 1 molal activity.

Having obtained the value of the free energy of formation of the ion at 1 molal activity at temperature T by means of equation (19) we can then obtain its free energy at any other concentration by applying equation (13).

It is more convenient to express this free energy change in terms of the total concentration of the substance in all of its forms and the hydrogen ion concentration of the solution. Let us consider a divalent substance. Its total concentration in all of its forms is

The ionization relations are given by the equations

and

$$H_{2}S \rightarrow HS^{-} + H^{+}; \qquad HS^{-} \rightarrow H^{+} + S^{-}$$

$$\frac{(HS^{-})(H^{+})}{H_{2}S} = K_{1}; \qquad \frac{(S^{=})(H^{+})}{(HS^{-})} = K_{2}$$

$$(HS^{-}) = \frac{(S^{=})(H^{+})}{K_{2}}; \qquad (H_{2}S) = \frac{(HS^{-})(H^{+})}{K_{1}} = \frac{(S^{=})(H^{+})^{2}}{K_{1}K_{2}}$$

$$S_{Total} = (S^{-}) \frac{(H^{+})^{2}}{K_{1}K_{2}} + \frac{(S^{=})(H^{+})}{K_{2}} + S^{-}$$

$$= (S^{-}) \left(\frac{(H^{+})^{2} + K_{1}(H^{+}) + K_{1}K_{2}}{K_{1}K_{2}}\right) \qquad (20)$$

From equation (20) the concentration of the divalent ion at any hydrogen ion concentration and total concentration of the substance in all of its forms is given by

$$(S^{-}) = S_{\text{Total}} \left(\frac{K_1 K_2}{(H^{+})^2 + K_1 (H^{+}) + K_1 K_2} \right)$$
 (21)

The free energy of dilution of this ion from the state at 1 molal activity to the concentration (S=) from equation (13) is

$$\Delta F = RT \ln \frac{(S^{-})}{1}$$

$$= RT \ln S_{Total} \left(\frac{K_1 K_2}{(H^{+})^2 + K_1 (H^{+}) + K_1 K_2} \right)$$
(22)

The free energy of the ion S= at any given hydrogen ion concentration and for any given total concentration of the electrolyte from equation (19) then is

$$\Delta F_{S^-,(S^-),T} = \Delta F^{\circ}_{\text{(neutral molecule,1M,T)}} - RT \ln K_1 K_2$$

$$-RT \ln S_{\text{Total}} \left(\frac{K_1 K_2}{(H^+)^2 + K_1(H) + K_1 K_2} \right)$$
(23)

Equation (23) is used in computing oxidation-reduction potentials from thermal data.

(7) Free Energy and Electromotive Force. The free energy change may be expressed in terms of the electromotive force generated by a cell operating under thermodynamically reversible conditions. The relation between these two quantities is

$$\Delta F = -Enf \tag{24}$$

The sign of E is taken as positive when the reaction proceeds spontaneously, n is the number of electrochemical equivalents involved in the reaction, f is the Faraday equivalent. ΔF is usually expressed as calories. The energy units of the right hand member of equation (24) are volt-coulombs. The factor converting calories to volt-coulombs is 4.18.

The variation of E with temperature is dependent upon ΔH in the same manner as ΔF in equations (11) and (12). The relation here, known as the Gibbs-Helmholtz equation, is

$$nfd\left(\frac{E}{T}\right) = \frac{\Delta H}{T^2} dT \tag{25}$$

In the integrated form, with the same assumptions as in equation (12), this equation becomes

$$-nf\left(\frac{E_2}{T_2} - \frac{E_1}{T_1}\right) = \Delta H\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$
 (26)

(8) Free Energy Values from Thermal Data. A great extension of the usefulness of thermodynamics in chemistry and physiology was obtained when it became possible to obtain free energy values by exclusively thermal measurements. Until then free energy data were obtainable only from equilibrium studies. There are several very important practical limitations to equilibrium methods. It is frequently extremely difficult or even impossible to obtain a system of which the substance whose free energy is desired is a component, and is present in the equilibrium state of the system in a measureable concentration. Furthermore, the reaction whose equilibrium is being measured is often only guessed at. The great advantages of the thermal method are that we are not restricted to perfectly (thermodynamically) reversible systems; and the reaction, for which the free energy change is desired, can always be formulated precisely, without ambiguity or assumptions. Furthermore the actual thermal measurements can now be made with high precision. For these reasons the free energy values based on thermal data have become the final reference values. This is especially true of organic compounds.

The determination of free energy values by thermal methods is based on the equation

$$\Delta F = \Delta H - T \Delta S \tag{27}$$

This equation applies only to isothermal processes. The two quantities in equation (27) which are determined experimentally are ΔH and ΔS . We have already described the manner in which ΔH may be obtained by measuring the heat of combustion. Let us again take glycine as an example to illustrate the determination of ΔS , the entropy of formation. Its formation may be represented as an hypothetical reaction

$$2C_{(graphite)} + \frac{5}{2}H_{2(gas)} + \frac{1}{2}N_{2(gas)} + O_{2(gas)} \rightarrow CH_2(NH_2) \cdot COOH \text{ (solid)}$$

The entropy of formation of glycine, ΔS_T , at the temperature T is given by the equation

$$\Delta S_T = S_{(glycine,T)} - 2S_{(C,T)} - \frac{5}{2}S_{(H_2,T)} - \frac{1}{2}S_{(N_2,T)} - S_{(O_2,T)}$$
(28)

According to the third law of thermodynamics the entropy of a compound may be determined by the relation

$$S_{\text{(compound},T)} = \int_0^T \frac{C_P}{T} dT + \frac{\Delta H}{T_{tr}}.$$
 (29)

 C_P is the specific heat of the substance at constant pressure and ΔH is here the change in heat content in the transitions which it may undergo(i.e., changes in the form of the compound) in warming the substance from 0° K to T° K and T_{tr} is the temperature of the transition.

Values of C_P are measured from as low a temperature as possible to the temperature T. The integral is then obtained graphically with the employment of an empirical extrapolation from the lowest temperature at which C_P has been measured to the absolute zero. The entropies of the elements have been obtained in the same way. The values of some of these are given in Table I. In recent years spectroscopic data have also been used for the calculation of entropy values (1). These values are more reliable for very simple compounds than those which are obtained from specific heats.

Table I

Entropies of Elementary Substances

***************************************	Element		$S_{298.1}$	
	C (graphite)		1.36	
	H ₂ (gas)		31.23	
	N ₂ (gas)		45.78	
	O ₂ (gas)		49.03	
	S (orthorhomb	ie)	7.64	

(Parks, G. S., and Jacobs, C. J., J. Amer. Chem. Soc., 56, 1513 (1934); Kelley, K. K., U. S. Bureau of Mines Bulletin No. 350, (1932).)

Once the entropies of the compound and of its constituent elements have been obtained, ΔS_T , the entropy of formation of the compound, can be computed; and this value inserted into equation (27) together with the value of ΔH , obtained as a rule from the heat of combustion, gives the value of ΔF , the free energy of formation of the substance in a specified standard state, at the temperature T. This value we shall designate as ΔF_T °. ΔS_T °, and ΔH_T ° have an analogous significance. The values of these quantities at 25°, i.e., $\Delta S_{298.1}$ °, $\Delta H_{298.1}$ °, and $\Delta F_{298.1}$ °, etc. for a number of compounds are given in Table II.

TABLE II

Entropies, Heats of Formation, and Free Energies of Formation of Some Amino Acids and Related Substances

Substance	Formula	Mol. Wt	Entropy at 25° S _{298,1} E.U.	Entropy of Formation at 25° $\Delta S_{298.1}$ E.U.	Heat of Combus- tion at 25°	Heat of Formation at 25° ΔH° _{298.1}	Free Energy of Formation at 25° $\Delta F^{\circ}_{298\cdot1}$
					Calories	Calories	Calories
					per mole	per mole	per mole
d-Alanine	C ₂ H ₇ O ₂ N	89.093	31.6	-153.7	387,210	-134,600	- 88,780
d, l -Alanine	C ₂ H ₇ O ₂ N	89.093	31.6	-153.7	386,620	-135,190	- 89,380
l-Asparagine (anhydrous).	C ₄ H ₈ O ₅ N ₂	132,118	41.7	-208.0	460,850	-189,360	-127,360
l-Asparagine (hydrate)	C4H10O4N2	150.134	51.0	-254.4	458,130	-260,390	-184,560
l-Aspartic Acid	C ₄ H ₇ O ₄ N	133.103	41.5	-194.2	382,720	-233,330	-175,440
Benzoic Acid	C7H6O2	122.117	40.8	-111.4	770,880	- 93,740	- 60,520
Carbon Dioxide (gas)	CO ₂					-94,240	-94,100
Creatine (anhydrous)	C4H ₂ O ₂ N ₂	131.134	45.3	-218.4	555,370	-129,000	- 63,830
Creatine (hydrate)	C4H11O2N2	149.150	56.0	-263.4	552,350	-200,330	-121,810
Creatinine	C ₄ H ₇ ON ₈	113.119	40.0	-167.9	558,450	- 57,600	- 7,550
l-Cysteine	C ₂ H ₇ O ₂ NS	121.12	40.6	-152.3	532,200	-127,880	-82,480
<i>l</i> -Cystine	C ₆ H ₁₂ O ₄ N ₂ S ₂	240.23	68.5	-286.1	997,770	-251,920	-166,630
Glycine	C ₂ H ₅ O ₂ N	75.067	26.1	-126.6	232,600	-126,660	- 88,920
Hippuric Acid	C ₂ H ₂ O ₂ N	179.168	57.1	-192.1	1,007,860	-147,710	- 90,440
Fumaric Acid	C ₄ H ₄ O ₄	116.071	39.7	-126.3	318,710	-194,880	-157,230
d-Glutamic Acid	C ₆ H ₉ O ₄ N	147.128	45.7	-222.6	537,450	-241,160	-174,800
l-Leucine	C6H12O2N	131.169	49.5	-233.6	856,090	-153,390	- 83,750
d-Leucine	$C_6H_{12}O_2N$	131.169	49.5	-233.6	856,110	-153,360	- 83,720
d,l-Leucine	C6H13O2N	131.169	49.5	-233.6	855,320	-154,160	- 84,520
d,l-Leucylglycine	C8H16O2N2	188.221	67.2	-312.8	1,093,330	-207,100	-113,850
l-Tyrosine	C ₂ H ₁₁ O ₂ N	181.184	53.0	-227.4	1,058,450	-165,430	- 97,640
Urea	CH.ON2	60.057	25.2	-108.9	150,990	- 79,870	- 47,410
Uric Acid	C.H.O.N.	168.113	41.4	-193.0	459,120	-148,710	- 91,180
Water (liq.)	H₂O	18.016	1000			- 68,310	- 56,720

(Huffman, H. M., Ellis, E. L., and Fox, S. W., J. Amer. Chem. Soc., 58, 1728 (1936).)

The methods by which the free energy of a compound in solution may be calculated from ΔF_T° has been described above.

4. APPLICATIONS OF THE FIRST LAW OF THERMODYNAMICS

The usefulness of thermodynamics in biochemistry and physiology, for the time being at any rate, is in the application of its

methods and data to specific systems. We shall (and may) take for granted the validity of the first and second laws of thermodynamics in biological systems.

We shall now consider a number of specific applications of the foregoing principles and equations. The order in which these are presented will in general follow the development of methods for obtaining the heat contents and free energies of amino acids and peptides in solution, and the application of these values to some questions of physiological interest.

(1) Heats and Free Energies of Formation of Pure Amino Acids. In Table II are collected values for S, ΔS , the heats of combustion, ΔH , and ΔF , of a number of amino acids in their standard states. The values for a few other related compounds are also included. The free energy values for the amino acids were obtained by means of equation (27) and are based upon thermal data—specific heats and heats of combustion. Obtained in this way, the accuracy of the free energy values is limited by the absolute accuracy (not percentage accuracy) of the values for the heats of combustion. The error in the heats of combustion given in Table II is not greater than ± 0.03 per cent.

The errors in nearly all of the older combustion values in the literature are much greater than this. This was the result in part of inadequate experimental technique, and in part to too low a degree of purity in the specimens which were used. In order to obtain a combustion value whose error is not greater than ± 0.03 per cent the compound must be prepared in an extraordinarily high degree of purity. Furthermore the form of the specimen burned must be defined. For example, the heats of combustion of two different crystal forms of anhydrous l-asparagine differ by 3300 calories, and in the case of anhydrous creatine by 500 to 600 calories. More examples will doubtless be found. The values given in Table II are those for the stable forms at 25°. An analogous difference in the solubilities of different crystal forms of amino acids have been observed.

Another even more important precaution must be borne in mind in using combustion values. The reaction must be precisely defined. It must be ascertained whether the value given refers, for example, to the reaction at constant pressure or at constant volume. In the case of sulfur compounds the value will be significantly different according to the final state assigned to the sulphur after the combustion. The same necessity for taking into account the final

state of each of the products of combustion applies, of course, to the other elements. In subtracting the combustion value of one compound from that of another, (or their heats of formation) it is essential that the combustion values in each case refer to precisely defined initial states and to the same states of the final products. It is because these precautions were omitted that most of the older data on the combustion of amino acids are of little value in their application to physiological questions.

We shall take l-aspartic acid as an example. It has been demonstrated that this substance participates in the reaction fumarate $+NH_4+\rightarrow l$ -aspartate. The heat of combustion of l-aspartic acid at constant pressure and at 25° is 382,720 calories. An error of 1 per cent in this value -3800 calories, is carried directly into its free energy value. Hence the equilibrium constant calculated from thermal data would be changed from 0.002 to nearly 1.0, the former being the observed experimental value.

The specific heat data do not, for our present purposes, call for the same high degree of absolute accuracy as do the combustion values. The error in the figures given in Table II for ΔS is of the order of magnitude of ± 1 entropy unit. This introduces an uncertainty of ± 300 calories in the free energy values.

- (2) Heat Contents of Solutions. Our next task is to evaluate the heat contents and free energies of amino acids in aqueous solution. We shall consider first the heat contents. The theoretical treatment will be more elaborate than the data available at present warrant; but it will indicate the types of experiments which are useful in this connection, the methods by which a given value can be obtained in several ways, and their relative reliability for the determination of this value.
- (3) Partial Molal Heat Content. Let us consider H, the heat content of a binary solution at constant temperature and pressure. The following considerations will apply to any other extensive property of this solution, its volume, specific heat, free energy and so on. In the addition of dn_2 moles of solute, the heat content will be increased by dH.

$$\frac{\partial H}{\partial n_2} = \overline{H}_2 \tag{30}$$

 \overline{H}_2 defined in this way is the partial molal heat content of the solute for this particular solution consisting of n_1 moles of solvent

and n_2 moles of solute. The partial molal heat content in the standard solution is \overline{H}_2° . We shall call $\overline{H}_2 - \overline{H}_2^{\circ}$ the relative partial molal heat content of the solute in any given solution. $H_2(\text{solid}) - \overline{H}_2^{\circ}$ is a similar quantity for the solute in the solid form, and $\overline{H}_1 - \overline{H}_1^{\circ}$ for the solvent.

(4) The Relative Partial Molal Heat Content. While such extensive properties as the volume and the heat capacity can be measured directly and hence can be given absolute values we cannot state the absolute value of the total heat content of a solution. We can only measure the difference between its heat content and that of another solution. In order to compare one solution with another we can state the differences between their heat contents and that of the same standard solution. Hence it is desirable to obtain numerical values for $(\overline{H}_2 - \overline{H}_2^{\circ})$, and $(\overline{H}_1 - \overline{H}_1^{\circ})$.

From the general partial molal equation, the total heat content of any solution is

$$H = n_2 \overline{H}_2 + n_1 \overline{H}_1 \tag{31}$$

H may be defined by means of another quantity, by the equation

$$\phi_h = \frac{H - n_1 \overline{H}_1^{\circ}}{n_2} \tag{32}$$

 ϕ_h is the apparent molal heat content of the solute. Stating equation (32) in words: the apparent molal heat content of the solute in any given solution is the total heat content of the solution minus the product of the number of moles of solvent and its partial molal heat content in the standard state, i.e., at infinite dilution, divided by the number of moles of solute.

From equation (32)

$$H = n_2 \phi_h + n_1 \overline{H}_1^{\circ} \tag{33}$$

We shall consider the energy relations in the following reaction

$$x_2X_2$$
 (solid) $\rightarrow n_2X_2$

 $\Delta H/n_2$ for this relation is the gain in heat content per mole of solute when it is dissolved in a solution in which the molality is n_2X_2 . It is equal to $(\overline{H}_2-H_{2(\text{solid})})$. A derivation of this relation, although approximate, may make it clearer.

$$dn_2X_2$$
 (solid) + $(m-dn_2)X_2 \cdot n_1H_2O \rightarrow mX_2n_1H_2O$

From our having made dn_2 very small we shall assume that \overline{H}_1 and \overline{H}_2 are the same in the initial and final states of the solution.

$$d\Delta H = m\overline{H}_2 + n_1\overline{H}_1 - dn_2H_{2\,(\text{solid})} - m\overline{H}_2 + dn_2\overline{H}_2 - n_1\overline{H}_1 = dn_2(\overline{H}_2 - H_{2\,(\text{solid})})$$

Therefore $d\Delta H/dn_2$, which is the partial molal heat of the solute in the given solution is $(\overline{H}_2-H_{2(solid)})$.

It is inconvenient to obtain an experimental measurement of $(\overline{H}_2-H_{2(solid)})$ by this direct method. We can obtain it indirectly from two other quantities which can be determined experimentally, the heat of solution of the solute in an infinitely dilute solution, and the heat of dilution.

(5) Heat of Solution at Infinite Dilution. We may write the first of these two reaction as follows.

$$n_2 X_2 \text{ (solid)} + \infty \text{ H}_2 \text{O} \rightarrow n_2 X_2 \infty \text{ H}_2 \text{O}$$
$$\Delta H^{\circ} = n_2 \overline{H}_2^{\circ} + \overline{H}_1^{\circ} - n_2 H_{2 \text{(solid)}} - \infty \overline{H}_1^{\circ} = n_2 (H_2^{\circ} - H_{2 \text{(solid)}})$$

Therefore the heat of solution per mole of solute in an infinitely dilute solution is

$$\frac{\Delta H^{\circ}}{n_2} = (\overline{H}_2^{\circ} - H_{2(\text{solid})})$$

The heat of the solution of the solute in a solution at infinite dilution is related to its heat of solution at any specified molality by the equation

$$(\overline{H}_2 - H_{2(\text{solid})}) = (\overline{H}_2^{\circ} - H_{2(\text{solid})}) + (\overline{H}_2 - \overline{H}_2^{\circ})$$
(34)

(6) The Heat of Dilution. The value of the second term on the right hand side of this equation is not obtained as directly, either experimentally or theoretically, as $(\overline{H}_2^{\circ} - H_{2(\text{solid})})$. It is, of course, related to the heat of dilution.

We may write the reaction for the dilution of any given solution

$$n_2X_2 \cdot n_1H_2O + (\infty - n_1)H_2O \rightarrow n_2X_2 \infty H_2O$$

The total heat content of the solution in its final state

$$H^{\circ} = n_2 \overline{H}_2^{\circ} + \infty \overline{H}_1^{\circ}$$

Similarly the heat content of the solution in its initial state is

$$H' = n_2 \overline{H}_2 + n_1 \overline{H}_1$$

and the heat content of the added water is

$$H^{\prime\prime} = \infty \overline{H}_1^{\circ} - n_1 \overline{H}_1^{\circ}$$

The increase in heat content in this reaction

$$H^{\circ} - H' - H'' = \Delta H = n_2 \overline{H}_2^{\circ} + \infty \overline{H}_1^{\circ} - n_2 \overline{H}_2 - n_1 \overline{H}_1 - \infty \overline{H}_1^{\circ} + n_1 \overline{H}_1^{\circ}$$

$$\therefore \Delta H_{\text{dilution}} = n_2 (\overline{H}_2^{\circ} - \overline{H}_2) + n_1 (\overline{H}_1^{\circ} - \overline{H}_1)$$
(35)

From the point of view of equation (31) H° , H', and H'' in the above solutions are

$$H^{\circ} = n_{2}\phi_{h}^{\circ} + \infty \overline{H}_{1}^{\circ}$$

$$H' = n_{2}\phi_{h} + n_{1}\overline{H}_{1}^{\circ}$$

$$H'' = \infty \overline{H}_{1}^{\circ} - n_{1}\overline{H}_{1}^{\circ}$$

$$\therefore \Delta H_{\text{dilution}} = (\phi_{h}^{\circ} - \phi_{h})$$
(36)

From equations (35) and (36)

$$n_2(\phi_h^{\circ} - \phi_h) = n_2(\overline{H}_2^{\circ} - \overline{H}_2) + n_1(\overline{H}_1^{\circ} - \overline{H}_1)$$

$$\therefore \text{ Per mole of solute } (\phi_h^{\circ} - \phi_h) = (\overline{H}_2^{\circ} - \overline{H}_2) + \frac{n_1}{n_2} (\overline{H}_1^{\circ} - \overline{H}_1)$$
 (37)

 $(\phi_h^{\circ} - \phi_h) = -$ (The relative apparent molal heat content of the solute), is an experimental quantity. It is the negative of the heat of dilution of a given solution to infinite dilution.

We must now derive an expression involving $(\phi_h^{\circ} - \phi_h)$ which will lead to evaluation of $(\overline{H}_2^{\circ} - \overline{H}_2)$ and $(\overline{H}_1^{\circ} - \overline{H}_1)$.

Differentiating equation (36) with respect to n_2

$$\frac{d(\Delta H_{\text{dilution}})}{dn_2} = (\phi_h^{\circ} - \phi_h) + n_2 \frac{d(\phi_h^{\circ} - \phi_h)}{dn_2}$$

$$= \frac{dH^{\circ}}{dn_2} - \frac{dH'}{dn_2} - \frac{dH''}{dn_2}$$
(38)

which from our definition of the partial molal heat content in equation (3)

$$\frac{d(\Delta H_{\text{dilution}})}{dn_2} = \overline{H}_2^{\circ} - \overline{H}_2 \tag{39}$$

Combining equations (38) and (39)

$$(\overline{H}_2^{\circ} - \overline{H}_2) = (\phi_h^{\circ} - \phi_h) + n_2 \frac{d(\phi_h^{\circ} - \phi_h)}{dn_h}$$

$$\tag{40}$$

Equation (40) is Rossini's (2) expression of the value of $(\overline{H}_2^{\circ} - \overline{H}_2)$ in terms of the molal heat of dilution and the derivative of this quantity with respect to the molality of the solute.

In order to evaluate $(\overline{H}_1^{\circ} - \overline{H}_1)$ in similar terms we employ equation (37) from which

$$(\overline{H}_1^{\circ} - \overline{H}_1) = \frac{n_2}{n_1} (\phi_h^{\circ} - \phi_h) - \frac{n_2}{n_1} (\overline{H}_2^{\circ} - \overline{H}_2)$$

$$\tag{41}$$

Substituting in this equation the expression for $(\overline{H}_2^{\circ} - \overline{H}_2)$ in equation (40)

$$(\overline{H}_{1}^{\circ} - \overline{H}_{1}) = \frac{n_{2}}{n_{1}} (\phi_{h}^{\circ} - \phi_{h}) - \frac{n_{2}}{n_{1}} \left[(\phi_{h}^{\circ} - \phi_{h}) + n_{2} \frac{d(\phi_{h}^{\circ} - \phi_{h})}{dn_{2}} \right]$$

$$= -\frac{n_{2}^{2}}{n_{1}} \frac{d(\phi_{h}^{\circ} - \phi_{h})}{dn_{2}}$$
(42)

Where values of n_2 are given per 1000 grams of water $n_1 = 55.51$ and equation (42) becomes

$$(\overline{H}_1^{\circ} - \overline{H}_1) = -\frac{n_2^2}{55.51} \frac{d(\phi_h^{\circ} - \phi_h)}{dn_2}$$
 (43)

- (7) Experimental Values of Heats of Dilution of Amino Acids. In Table III data are presented on the heats of dilution of some amino acids obtained by Zittle and Schmidt (3). The figures in this table are somewhat different from those given by these authors. They were obtained from smooth curves drawn through their actual experimental values. Empirical equations were fitted to these curves; and the values of $d(\phi_h \phi_h^{\circ})/dm$ were obtained by differentiation. This procedure, and a somewhat different weighting of the data are responsible for the small differences between the values in Table III and those given by Zittle and Schmidt.
- (8) Heat of Dilution of the Solvent. The value of the heat of dilution of the solute may be obtained indirectly by measuring the heat of dilution of the solvent. The experimental procedure here is to transfer a weighed quantity of water into such an amount of a solution of known molality that the final molality is essentially unchanged. This experimental approximation will be introduced into the equation for the heat of the reaction. The reaction may be written

$$n_2 X_2 \cdot n_1 - dn H_2 O + dn H_2 O \rightarrow n_2 X_2 \cdot n_1 H_2 O$$

$$\Delta H = dn (\overline{H}_1 - \overline{H}_1^{\circ})$$
(44)

from which the heat of dilution per mole of added solute is $(\overline{H}_1 - \overline{H}_1^{\circ})$. The heat of reaction per mole of water added in the above reaction

TABLE III

Heats of Dilution $(\phi_h - \phi_h^{\circ})$ and Relative Molal Heat Contents $(\overline{H}_2 - \overline{H}_2^{\circ})$ of Amino Acids In Calories, In Aqueous Solution at 25°

Amino Acid	A	В	C	D	E	F	Molality of Sat'd Sol'n	$\overline{H}_2 - \overline{H}_2^{\circ}$ for Satd Sol'n
d,l-Alanine. d-Arginine t-Aspartic Acid. d-Glutamic Acid. Glycine. l-Histidine. t-Hydroxyproline. d-Lysine. t-Proline. d-Pyroglutamic acid d,l-Serine Taurine. d,l-Valine.	56.5 - 532 -2560* -2560* - 162.2 - 105 - 7 275 107.4 - 80.3 - 150* - 255 225	434 47.4 -5.48	-142 - 5.8	113 -1064 -5120 -5120 - 324.4 - 210 - 14 550 215 - 160.6 - 300 - 510 450	1300 142.2 -16.5	-568 - 23.2 0.48	1.88 (1.0)† 0.0376 0.0585 3.33 (0.5)† 2.75 (1.0)† (8.0)† (7.0)† 0.48 0.84 0.63	210 - 330 - 190 - 300 - 360 - 105 - 40 - 550 - 910 - 1120 - 140 - 240 - 280

$$(\phi_h - \phi_h^{\circ}) = Am + Bm^2 + Cm^3$$

$$(H_1 - H_2^{\circ}) = (\phi_h - \phi_h^{\circ}) + m. d \left(\frac{\phi_h - \phi_h^{\circ}}{dm}\right) = Dm + Em^2 + Fm^3$$

* Based on the single value of the saturated solution.

† These molalities are less than those of the saturated solutions. The $(\overline{H}_2 - \overline{H}_2^{\circ})$ values are calculated for these molalities.

(Zittle, C. A., and Schmidt, C. L. A., J. Biol. Chem., 108, 161 (1935).)

gives directly $(\overline{H}_1 - \overline{H}_1^{\circ})$. This value can be used to obtain $(\overline{H}_2 - \overline{H}_2^{\circ})$ by means of the equation

$$\int_{n_0=0}^{n_2=m} d(\overline{H}_2 - \overline{H}_2^{\circ}) = \int_{n_0=0}^{n_2=m} \frac{n_1}{n_2} d(\overline{H}_1 - \overline{H}_1^{\circ})$$
 (45)

TABLE IV

Heat of Dilution $(\phi_h - \phi_h^{\circ})$ of Glycine at 25°

Concentration (Moles)	Measured Directly Calories per mole	Calculated Calories per mole
0.02	0*	
0.04	2^*	
0.10	12*	25
0.33	45*	$oxed{52}$
0.50	70*	68
1.00	125*	126
3.00	210	235
3.33	225	250

* Measured at 18°. The order of magnitude of the temperature correction is for example for the 1 molal solution 0.5 calories per degree. In more dilute solutions the change is less.

(Zittle, C. A., and Schmidt, C. L. A., J. Biol. Chem., 108, 161 (1935).).

The right hand term of equation (45) is evaluated by graphical integration of the area under the curve obtained when n_1/n_2 is plotted as ordinate against $(\overline{H}_1 - \overline{H}_1^{\circ})$ as abscissa. The integral heat of dilution per mole of solute is then obtained by means of equation (37).

In Table IV values of the integral heat of dilution of glycine solutions calculated by this method are compared with those measured directly.

- (9) Differences Between Heat of Solution in an Actual Solution, and the Heat of Solution in an Ideal Solution. The last column in Table III gives the difference between the heat of solution of the solute per mole of solute in the actual saturated solution and the heat of solution which would have been observed had the solute formed a perfect solution in the saturated solution. The derivation of this relation is as follows. The reactions in the two cases are
- (1) $dnX_{2\text{(solid)}} + (m-dn)X_2 \cdot n_1 H_2 O \rightarrow mX_2 \cdot n_1 H_2 O$
- (2) $dnX_{2(\text{solid})} + (m-dn)X_2^{\circ} \cdot n_1 H_2O \rightarrow mX_2^{\circ} \cdot n_1 H_2O$

In computing the increase in heat content we have made dn so small compared with m that the composition of the initial and final solutions are for practical purposes the same. In reaction (2) the saturated solution is perfect and hence the partial molal heat contents of the solute and solvent are \overline{H}_2° and \overline{H}_1° . For reaction (1)

$$\Delta H = dn(\overline{H}_2 - \overline{H}_{2(\text{solid})})$$

and for reaction (2)

$$\Delta H^{\circ} = dn(\overline{H}_{2}^{\circ} - \overline{H}_{2(\text{solid})})$$

$$\therefore \quad \Delta H - \Delta H^{\circ} = dn(\overline{H}_{2} - \overline{H}_{2}^{\circ})$$
(46)

Hence per mole of solute the difference between the two heats of solution is $(\overline{H}_2 - \overline{H}_2^{\circ})$. This value is useful in estimating the accuracy of the activity coefficients applied to the solute in the saturated solution in the calculation of the free energy of solution. This will be shown below.

From the determined values of $(\overline{H}_2^{\circ} - \overline{H}_{2(\text{solid})})$ and $(\overline{H}_2 - \overline{H}_2^{\circ})$ we obtain $(\overline{H}_{2(\text{sat'd})} - \overline{H}_{2(\text{solid})})$, the molal heat of solution of the solute into the saturated solution. These values for the amino acids for which data are available are collected in Table V.

For comparison we have collected in Table VI values of $(\overline{H}_2 - \overline{H}_2^\circ)$ for sodium chloride and amino acids in aqueous solution at 25°. These figures show that, except for l-aspartic and l-glutamic acids,

Table V

Calculated and Observed Values of Heats of Solution of Amino Acids in Aqueous Solution at 25°

	$(H_2-H_2^{\circ})$	(solid)		(H	7 _{2 (sat'd)} -H _{2 (}	solid)
Amino Acid	Measured directly	Calculated from solubility data	$(H_{2 \text{ (sat'd)}} - \bar{H}_{2}^{\circ})$ Measured	From thermal data	Calculated from solu- bility and activity coefficients	$\frac{d \ln \gamma}{d \ln m}$
d-Alanine		1830			1830	0
d.l-Alanine	2040 ± 20	2200	210	2250	2200	Ö
d-Arginine	1500		- 425 (1.0M)	1100		
l-Asparagine · H ₂ O	8000	8430		8000		
l-Asparagine	5750			5750		
l-Aspartie acid	6000	6180	- 200?	5800	5580	-0.549
d.l-Aspartic acid	7100	7160	- 200?	6900	6500	-0.549
l-Cystine		5510				
I-Diiodotyrosine		7820			7830	0
d-Glutamic acid		6540		6300	6050	-0.539
d.l-Glutamic acid	6530	6170	- 200?	6330	5710	-0.539
Glycine	3750 ± 15	3370	- 400	3350	3370	-0.0607
l-Histidine	3300		- 90 (0.5M)	3200	1 1 1 1 1 1	
l-Hydroxyproline	1400	1440	- 35 (2M)	1400		
d-Isoleucine		840				
d,l-Isoleucine		1780			1780	0
l-Leucine		840		100	830	0.382
d.l-Leucine		2000			2070	0.382
d-Lysine	-4000 ± 100		500 (1M)	-3500		
d,l-Methionine	4000 ± 100	4230		4000		
d.l-Norleucine		2530			2530	0
l-Phenylalanine		2820				
1.1-Phenylalanine		2760			2760	0
l-Proline	-750 ± 50	1340	1050 (8M)	> 300		
d-Pyroglutamic acid.	3600 ± 40		-1100 (7M)	2500		
d,l-Serine	5180 ± 60	5410	- 130	5050		
Taurine	6000 ± 100	5980	- 300	5700		
l-Tryptophane		1360		100		
l-Tyrosine		5950			5950	0
d-Valine		500				
<i>d,l-</i> Valine	1430 ± 25	1460	300	1730	1590	-0.0549

(The data were taken from Dalton, J. B., and Schmidt, C. L. A., J. Biol. Chem., 103, 549 (1933); 109, 241 (1935); J. Gen. Physiol., 19, 767 (1936); Tomiyama, T., and Schmidt, C. L. A., J. Gen. Physiol., 19, 379 (1935); Zittle, C. A., and Schmidt, C. L. A., J. Biol. Chem., 108, 161 (1935), and recalculated as indicated in the text.)

the amino acids form in dilute solutions more perfect solutions than a typical mono-monovalent strong electrolyte. In higher concentrations this difference disappears. A better expression of the imperfection of the solution than $(\overline{H}_2 - \overline{H}_2^{\circ})$ is the integral heat of dilution, $(\phi_h - \phi_h^{\circ})$; this quantity takes into account the state of both solute and solvent.

The figures in Table VI show that for concentrations of amino acids below 1.0 molal the absolute quantities of heat measured are quite small. A very high degree of precision in the calorimetry is required before these values can be given more than qualitative

significance. Furthermore it is not permissible to extrapolate far to zero concentration. The figures in the case of sodium chloride show that the sign of the heat effect may change. For this reason there are large discrepancies in the literature for the values for $(\overline{H}_2 - \overline{H}_2^{\circ})$ even for sodium chloride in dilute solution.

(10) Heats of Hydration of l-Asparagine and of Creatine. The

Table VI

Comparison of Relative Partial Molal Heat $(\overline{H}_2-\overline{H}_2^\circ)$ Contents (In Calories) of Sodium

Chloride and Amino Acids in Aqueous Solution at 25°

M	0.01	0.05	0.1	0.6	1.0	2.0	3.0
Activity coefficient of NaCl aq. 25°	0.922	0.842	0.798		0.650	0.661	0.704
NaCl	64	94	102	-48	-188	-466	-626
d,l-Alanine	1	6	11	68	113	240	
d-Arginine	-11	-20	-94	-280	-330		
l-Aspartic acid	-51	-260					
d-Glutamic acid	-51	-260					
Glycine	-3	-10	-31	-150	-205	-267	-310
<i>l</i> -Histidine	-2	-11	-21	-120			
l-Hydroxyproline	-0.1	-0.7	-1	-8	-14	-28	-42
d-Lysine	5.5	27.5	55	330	550		
<i>l</i> -Proline	2	11	22	123	200	368	510
d-Pyroglutamic acid	-2	-8	-16	-94	-160	-320	-480
d,l-Serine	-3	-15	-30	-180		48.7 (1) (1) (2) (1) (1) (1)	
Taurine	-5	-25	-48	-210	-240		
d,l-Valine	5	23	45	270			

(The data for the activity coefficient of NaCl were taken from Lewis, G. N., and Randall, M., Thermodynamics and the Free Energy of Chemical Substances, New York and London, 1923. The data for $\overline{H}_2 - \overline{H}_2$ °(NaCl) were taken from Robinson, A. L., J. Amer. Chem. Soc., 54, 1311 (1932). The other data were taken from Zittle, C. A., and Schmidt, C. L. A., J. Biol. Chem., 108, 161 (1935), and recalculated as indicated in the text.)

value for the heat of hydration of l-asparagine can be calculated from the $(\overline{H}_{2(\text{sat'd})} - \overline{H}_{2(\text{solid})})$ values in Table V. The reaction for the hydration at 25° may be written as follows:

l-Asparagine_(solid) $\rightarrow l$ -Asparagine_(aq., m=a); $\Delta H = 5750$ calories

 $\textit{l-}Asparagine \cdot H_2O_{(solid)} \rightarrow \textit{l-}Asparagine \cdot H_2O_{(aq.,\ m=a)};$

 $\Delta H = 8000 \text{ calories}$

l-Asparagine_(solid) + H₂O $\rightarrow l$ -Asparagine · H₂O; $\Delta H = -2250$ calories

Another independent method of obtaining the value of ΔH for this reaction is from the heats of formation. The values for the anhydrous and hydrated forms are given in Table II. We may write the reaction here

$$l$$
-Asparagine_(solid) + H₂O_(liquid) $\rightarrow l$ -Asparagine · H₂O_(solid)
 $\Delta H = -260,390 - (-189,360) - (-68,310) = -2720$ calories.

The difference between these two values is within the experimental error of either method.

The heat of hydration of creatine calculated from the heats of

TABLE VII

Specific Heat and Heat Capacity of Aqueous Solutions of Glycine, d,l-Alanine, and d,l-Valine at 25°

		Glye	ine			<i>d,l-</i> A1	anine			l,l-Va	line	
Molality	Specific Heat	C_{P_2}	Фс	C_{P_1}	Specific Heat	C_{P_2}	φσ	C_{P_1}	Specific Heat	C_{P_2}	φο	$C_{I\!\!P_1}$
M	Cal./(gm.				Cal./(gm.				Cal./(gm.			
	degree)				degree)		100		degree)			
0	0.998	7.5	7.5	17.98	0.998	40	40.0	17.98	0.998	93	93	17.96
0.2	0.985	8,5	7.5	17.98	0.988	39	39.5	17.98	0.993	84	90	18.01
0.5	0.965	9.0	8.0	17.97	0.974	37	38.0	17.99	0.983	69	85	18.09
0.63									0.976	63	82	18.15
1.0	0.936	11.5	8.5	17.94	0.950	34	37.0	18.04				
1.5	0.910	14.0	9.5	17.90	0.928	31	35.5	18.13			-	
1.88					0.910	29	35.0	18.16				
2.0	0.886	16.5	11.0	17.84								
2.5	0.867	19.5	13.0	17.73							A. I.	
3.0	0.851	22.0	15.0	17.66								
3.33	0.840	23.5	15.5	17.56							10.2	

(Zittle, C. A., and Schmidt, C. L. A., J. Biol. Chem., 108, 161 (1935).)

formation of the anhydrous and hydrated forms is similarly

$$\Delta H = -200,330 - (-129,300) - (-68,310) = -3020$$
 calories.

This value is nearly that for the hydration of l-asparagine.

(11) Partial Molal Heat Capacities. It is sometimes necessary, as for example in constructing a general equation for the free energy of solution over a range of temperatures, to determine the temperature coefficients of heats of solution, and of dilution.

The fundamental equation here is the Kirchhoff equation

$$\frac{d(\Delta H)}{dT} = \Delta C_P = \text{Specific heat of the products} - \text{specific heat of the}$$
reactants (47)

We shall require therefore the specific heats of the solid amino

acids and of their partial molal specific heats at different molalities in aqueous solution. The latter data for glycine, d,l-alanine, and d,l-valine were obtained by Zittle and Schmidt (Table VII).

In this table the experimentally measured thermal quantities are the specific heats of solutions of different concentrations of the amino acids, i.e., C_P for different values of m.

Analogous to equation (32) C_P is related to the apparent molal heat capacity of the solute, ϕ_C , by the equation

$$C_P = n_2 \phi_C + n_1 \overline{C}_{P_1}^{\circ} \tag{49}$$

Similarly the partial molal heat capacity of the solute is

$$\overline{C}_{P_2} = \frac{\partial C_P}{\partial n_2} \tag{50}$$

The values of \overline{C}_{P2} in Table VII were obtained from the plot of C_P as ordinate values against $m(n_2)$ as abscissa. The slopes of the curve at different values of m give \overline{C}_{P_2} directly.

The values for \overline{C}_{P_1} , the partial molal heat capacity of the solvent, were derived from the following relations

$$n_1 d\overline{C}_{P_1} + n_2 d\overline{C}_{P_2} = 0 \tag{51}$$

From which

$$\overline{C}_{P_1} = -\int_{n_2=0}^{n_2=m} \frac{n_2}{n_1} d\overline{C}_{P_2} + \overline{C}_{P_1}{}^{\circ}$$
 (52)

The value of the integral on the right hand side of equation (52) was obtained by plotting n_2/n_1 against \overline{C}_{P_2} (obtained as described above) and integrating graphically.

(12) Temperature Coefficient of Heat of Dilution and of Solution. With these values of \overline{C}_{P2} and \overline{C}_{P1} we obtain directly values of $d(\Delta H)/dT$ for such processes as dilution of a solution of given molality to infinite dilution, and of solution of the solid solute in an ideal solution. Thus for dilution of the solute the equation is

$$\frac{d(\overline{H}_{2}^{\circ} - \overline{H}_{2})}{dT} = \overline{C}_{P_{2}}^{\circ} - \overline{C}_{P_{2}}$$

$$(53)$$

It is understood that \overline{H}_2 and \overline{C}_{P_2} refer to a specific molality.

Similarly for solution of the solid solute in an ideal solution the equation is

$$\frac{d(\overline{H}_{2}^{\circ} - \overline{H}_{2(\text{solid})})}{dT} = \overline{C}_{P_{2}}^{\circ} - C_{P_{2}(\text{solid})}$$
(54)

We may assume that ΔC_P is constant for such processes as dilution and solution over the temperature range in which we shall use the data.

Equation (47) permits us to obtain a value of ΔH as a function of the temperature. The integrated form of this equation, taking ΔC_P as constant, is,

$$\Delta H = \Delta C_P T + C \tag{55}$$

To obtain the value of the constant of integration we may insert known values of ΔH and ΔC_P at any temperature T. If, for example, we wish to obtain the value of the heat of dilution as a function of the temperature we may take the value of $(\overline{H}_2^{\circ} - \overline{H}_2)$ for a given molality at, for example, 25°, and similarly, of $(\overline{C}_{P_2}^{\circ} - \overline{C}_{P_2})$ for this molality at this temperature. Though the $\overline{C}_{P_2}^{\circ}$ and \overline{C}_{P_2} will vary with temperature their difference will remain unchanged. Hence $d(\overline{H}_2^{\circ} - \overline{H}_2)/dT$ will be a constant.

The temperature coefficient of the integral heat of dilution, i.e., of the whole solution, per mole of solute is

$$(\phi_C^{\circ} - \phi_C) = \frac{n_1}{n_2} (\overline{C}_{P_1}^{\circ} - \overline{C}_{P_1}) + (\overline{C}_{P_2}^{\circ} - \overline{C}_{P_2})$$

$$(56)$$

The derivation of equation (56) is similar to that of equation (37). In using heat capacity data, as they are here, in order to plot curves from which the slopes are taken for the \overline{C}_{P_2} values, the measurements must be of the highest possible accuracy, and over as large a range as possible. The final values will depend to some extent on individual judgment. Thus on plotting the specific heat data of glycine, Table VII, against molality it will be seen that individual values for C_{P_2} may justifiably differ by as much as 4. This uncertainty is carried into the temperature coefficients. In the case of d,d-valine the data can be represented equally well within the experimental error (± 0.2 per cent) by a straight line. In this case C_{P_2} is constant and the temperature coefficient of the heat of dilution is zero instead of 11 as given by the data in Table VII.

The temperature coefficients of the heats of dilution, and of solution into ideal solutions are given in Table VIII. The value used for $C_{P(solid)}$ for glycine is 24.0 calories, and for d,l-alanine 29.0 calories. The other specific heat values (except those bracketed) are those given by Zittle and Schmidt.

For purposes of calculation in physiological experiments even large differences in the temperature coefficients are negligible. Thus a range of 12°, viz., from 25°, at which temperature most physiochemical data are now given, to 37°, the uncertainty in the case

Table VIII

Temperature Coefficients of the Heats of Dilution, and of Solution into Ideal Solution, of Glycine, d,l-Alanine, and d,l-Valine

	Tempera	ture Coeffi	cient of In	tegral Heat	of Dilution	$(\phi_c{}^{\circ}-\phi_c)$
Amino			al Heat	Temp	erature Coef	ficient
Acid	Initial Molality		n per mole bserved at 25°	Observed	Calculated from ϕ_c ° $-\phi_c$	Calculated from C _P Values
Glycine d,l-Alanine d,l-Valine	1.76	260 -112 -110	210 - 90 -105	-8.3 3.5 1	-7.5 5.0 10(0)	-8.6 5.0 15(0)

	Tempera		ient of $(\overline{H_2}^{\circ}\!-\!H_2)$ (solid)	
	$\overline{C_{P_2}}^{\circ}$	C_{P_2} (solid)	Value of Tempera- ture Coefficient from Specific Heats	Value of Temperature Coefficient from $d(\Delta H)/dT$ (see Table X)
Glycine d,l -Alanine d ,-Valine	40	24 29	-16.5 11	-14 15 31

The bracketed values for $(\phi_c^{\circ} - \phi_c)$ for d, l-Valine were obtained from another equally justifiable plot of the specific heat data.

(Zittle, C. A., and Schmidt, C. L. A., J. Biol. Chem., 108, 161 (1935).).

of the worst of these values, that of the heat of dilution of d,l-valine, amounts to only 180 calories.

5. APPLICATIONS OF THE SECOND LAW OF THERMODYNAMICS

(1) Free Energies of Dissolved Substances. In our development of methods for the calculation of the free energy of formation of amino acids in aqueous solution we shall first assume that the ratio of the mole fractions of the solute, at any two concentrations, is equal to the ratio of their thermodynamic activities.

The fundamental equation here is

$$\Delta F = RT \ln \frac{a^{\circ}}{a} \tag{57}$$

where a° is the activity in the standard state. This state has been defined as that in which 1 mole is dissolved in 1000 grams of water and forming an ideal solution, i.e., its activity coefficient is equal to unity.

In the case of a substance miscible with water in all proportions a convenient point of departure is the substance in the pure state. Its mole fraction in this state is unity. The reaction for the transfer of 1 mole of the solute from this state to a very large volume of an aqueous solution in which 1 mole is dissolved in 1000 grams of water may be written

$$X_{2(N=1)} \rightarrow X_{2(a_{1},N_{2}=0.0177)}; \Delta F = RT \text{ in } 0.0177$$

$$\therefore \Delta F_{(T,a_{1},N_{2}=0.0177)} = \Delta F_{T}^{\circ} + RT \text{ in } 0.0177.$$
(58)

In the case of a substance not miscible with water in all proportions our point of departure will be the saturated solution. Here $\Delta F^{\circ}_{(\text{solid})} = \Delta F_{(\text{saturated solution})}$. If the mole fraction in the saturated solution is N_2 then for the reaction

$$X_{2(\text{saturated solution})} \to X_{2(\text{aq.},N_2=0.0177)}; \Delta F = RT \ln \frac{0.0177}{N_2}$$

and hence

$$\Delta F_{(T,aq.,N_2=0.0177)} = \Delta F_T^{\circ} + RT \ln \frac{0.0177}{N_2}$$
 (59)

In Table IX are presented data on the solubility of amino acids obtained by Dalton and Schmidt (4) by Tomiyama and Schmidt (5) and by others. From the form in which these data are presented it is possible to calculate the heats of solution in the saturated solution and the temperature coefficient of this heat of solution.

When activity coefficients are introduced into free energy equations such as equation (57) the standard state is the same but the equation is usually given the form

$$\Delta F = RT \ln \frac{m^{\circ} \gamma^{\circ}}{m \gamma}$$

$$= RT \ln \frac{1}{m \gamma}$$
(60)

Table IX Coefficients of Solubility Equations of Certain Amino Acids in $Water^{1}$

Amino Acid	\$	$b_1 \times 10^2$	$c_1 \times 10^5$	a_2	a_3	$b_3 \times 10^2$	$c_3 \times 10^5$	a4	$b_4 \times 10^2$	c4×105
d-Alanine	2.1048	0.4669		0.1551	- 2.5792	1.075		-6.5150	1.037	
d L-Alanine	2.0830	0.5608		0.1333	-3.2199	1.291		-7.1317	1.245	
L-Asparagine H.O	0.9289	2.311	-4.981	-1.2475	-25.9584	1.159	-11.47	-30.2463	11.79	-11.84
LAspartic acid	0.3194	1.519		-1.8047	-13.7113	3.499		-17.7370	3.502	
d I-Aspartic acid	0.4181	2.016	-4.999	-1.7060	-25.1918	10.93	-11.51	-29.2797	10.98	-11.61
LCvstine	-1.299	1.357		-3.680	-18.643	3.125		-21.023	3.125	
d 1-Costines	-1.7959	0.8013	27.89	-4.1766	19.4912	-23.66	47.61	15.4747	-23.66	47.61
d I-Cystino ³	- 2.1087	3.367	-22.56	-4.4894	-36.9568	14.48	-16.80	-40.9733	14.48	-16.80
Meso-cystine	-1.7190	0.4514	27.39	-4.0997	34.7268	-33.38	63.01	30.7103	-33.38	63.01
Meso-cystine ³	-2.6034	5.890	-49.41	-4.9841	-133.4125	75.73	-113.8	-137.429	-75.73	-113.8
L-Dibromotyrosine										
(hydrated)	0.0839	1.627		-2.445	-15.881	3.753		-19.894	3.752	
l-Dibromotyrosine								1	,	
(anhydrous)	0.188	0.9884		-2.343	-11.610	2.276		-15.537	2.247	
L-Dichlorotyrosine	0.0065	1.038	4.648	-2.392	-4.058	-3.450	1.069	-8.426	-3.215	1.030
L-Dijodotyrosine	0690 -	1.92		-3.326	-19.745	4.42		-23.761	4.43	
d.l-Diiodotvrosine	-0.827	1.43		-3.464	-16.989	3.30		-21.006	3.30	
d-Glutamic acid	0.5331	1.613		-1.6345	-13.9054	3.714		-17.9095	3.709	
d.l-Glutamic acid	0.9317	1.523		-1.2359	-12.4244	3.507		-16.4071	3.495	
Glyging	2.1516	1.087	-4.114	0.2762	-13.2619	7.676	-9.473	-17.8976	8.171	-10.50
/-Hydroxyproline	2.4603	0.3891		0.3428	-1.6575	0.8959		-5.5906	0.8514	
d-Isolencine	1.5787	0.07682	2.594	-0.5389	2.7190	-3.081	5.972	-1.3913	-3.020	5.866
d.l-Isolencine	1.2616	0.2512	3.794	-0.8560	2.9651	-4.193	8.736	-1.1373	-4.134	8.632
L-Leneine4	1.3561	0.02233	3.727	-0.7615	4.5073	-4.683	8.582	-0.7252	-3.814	7.198
d L-Lengine	0.9013	0.2635	4.591	-1.2163	3.4260	-5.167	10.57	-0.6258	-5.143	10.53
d.l-Methionine	1.2597	1.108	1.221	-0.9140	-11.1682	4.086	-2.811	-15.2099	4.111	- 2.871
d,l-Norleucine	0.9258	0.4524	3.402	-1.1918	0.2523	- 3.236	7.833	-4.2067	-2.941	7.340
										Principal and the second

Table IX (Continued)

Amino Acid aı	$b_1 \times 10_2$	$c_1 \times 10^5$	a ₂	a_3	$b_3 \times 10^2$	c ₃ ×10 ⁵	<i>a</i> 4	b ₄ ×10 ²	c4×105
l-Phenylalanine	0.6982		- 0.9204	- 6.510	1.608		-10.5103	1.601	
d,l-Phenylalanine 0.9986	0.5252	3.140	-1.2192	-0.7184	-2.739	7.229	-5.0876	-2.495	808.9
<i>l</i> -Proline 3.1050	0.4206		1.0441	-0.2407	0.9686		-3.8586	0.7586	
<i>d,l</i> -Serine	1.520	-3.548	-0.6782	-17.2153	7.963	-8.169	-21.4529	8.134	-8.504
Taurine	1.916	-8.500	-0.5029	-27.8015	15.10	-19.57	-32.1283	15.35	-20.07
l-Tryptophane 0.9156	0.4834	2.988	-1.3942	-11.3824	4.872	6.881	-15.3928	4.869	-6.879
l-Tyrosine – 0.708	1.46		-2.966	-10.799	3.36		-20.062	3.37	
d-Tyrosine – 0.708	1.46		-2.966	-10.799	3.36		-20.062	3.37	
$d_{i}l$ -Tyrosine – 0.833	1.51		-3.091	-16.562	3.46		-20.577	3.46	
d-Valine ² 1.9211	8.1515	8.589	-0.1456	0.6274	- 8.927	1.978	-3.4570	- 8.528	1.9058
d-Valine ² 1.6675	97.75	80.22	-0.4011	-20.8468	123.4	18.47	-24.2293	119.4	17.85
<i>d</i> -Valine ² 1.8847	11.42	4.799	-0.1839	-0.3175	- 3.406	1.105	-3.6570	-8.125	1.910
<i>d</i> -Valine ² 1.9227			-0.1459	-0.3359			-4.3653		
d-Valine ² 1.8836			-0.1850	-0.4260			-4.4542		
d, l -Valine 1.7749	0.2389	2.607	-0.2966	-2.2921	- 2.729	6.003	-1.7417	- 2.705	5.928

¹ Solubility equations:

(a) Log $S = a_1 + b_1t + c_1t^2$ (grams per 1000 gms, water)

(b) Log $m=a_2+b_1t+c_1t^2$ (moles per 1000 gms. water)

(c) $m = a_3 + b_3 T + c_3 T^2$

(d) ln $N_2 = a_4 + b_4 T + c_4 T^2$

² The five sets of values which are given under d-valine refer to the various crystal forms.

³ The first set of values refer to 273.1° to 303.1° and the second set to 298.1° to 323.5° absolute. (Unpublished data of Campbell,

W. W., and Schmidt, C. L. A.)

4 Values are not strictly accurate due to contamination of the leucine with a small amount of methionine.

References: (Dalton, J. B., and Schmidt, C. L. A., J. Biol. Chem., 103, 549, (1933); 109, 241 (1935); J. Gen. Physiol., 19, 767 (1936); Tomiyama, T., and Schmidt, C. L. A., J. Gen. Physiol., 19, 379 (1935); Winnek, P. S., and Schmidt, C. L. A., J. Gen. Physiol., 18, 889 (1935); 19, 773 (1936); see also, Dunn, M. S., Ross, F. J., and Read, L. S., J. Biol. Chem., 103, 579 (1933).) m and γ are respectively the number of moles dissolved in 1000 grams of water, and the activity coefficient. In our hypothetical standard solution m° and γ° are each unity. For dilute solutions the numerical result is the same whether mole fractions are used or molalities. In more concentrated solutions such as the saturated solution of glycine, (M=3.33), there is a difference of 16 calories between RT ln 0.0177/0.0573 (RT ln 0.0177/ N_2) and RT ln 1/3.33 (RT ln 1/M). In the case of the saturated solution of urea, M=20.03, the difference is 170 calories. It would be better if all of the calculations of this type were made with mole fractions, as it is the more rigorous form.

(2) Determination of the Heat of Solution from Solubility Data. Assuming that the saturated solutions of amino acids are ideal solutions we can calculate the ideal heat of solution in the saturated solution as follows. The van't Hoff equation (11) is

$$d\!\!\left(\!\frac{\Delta F}{T}\!\right) = -\frac{\Delta H}{T^2} \, dT$$

The free energy change in transferring a mole of solute from the saturated solution to the standard solution, (substituting N_2 for the activity,) is, from equation (57),

$$\Delta F = RT \ln \frac{a^{\circ}}{N_2} \tag{61}$$

$$\therefore \frac{\partial \left(\frac{\Delta F}{T}\right)}{\partial T} = -R \frac{\partial \ln N_2}{\partial T} \tag{62}$$

Therefore from equation (11)

$$\frac{\partial \ln N_2}{\partial T} = \frac{\Delta H}{RT^2} \tag{63}$$

We obtain the value of $\partial \ln N_2/dT$ directly by differentiating with respect to T in equation (d) at the foot of Table IX. In Table V are given the values for ideal heats of solution for some of these amino acids calculated from the coefficients of the solubility equations. These values should be the same as $\overline{H}_2^{\circ} - \overline{H}_{2(\text{solid})}$. The differences represent uncertainties in the coefficients of the solubility equation, and in the experimental values.

By taking into account activity coefficients we can obtain from the solubility equations independent values for $(\overline{H}_{2(\text{sat'd})} - \overline{H}_{2(\text{solid})})$.

The necessary equation is derived as follows. For N_2 in equation (63) we substitute $m\gamma$. This equation then becomes

$$\frac{\partial \ln m\gamma}{\partial T} = \frac{\Delta H}{RT^2}$$

Hence

$$\frac{\partial \ln m}{\partial \ln T} + \frac{\partial \ln \gamma}{\partial T} = \frac{\Delta H}{RT^2} \tag{64}$$

and

$$\Delta H = RT^2 \left(\frac{\partial \ln m}{\partial T} \right) \left(1 + \frac{\partial \ln \gamma}{\partial \ln m} \right) \tag{65}$$

The values of $\partial \ln m/\partial T$ are obtained by differentiating with respect to T (in equation (c) at the foot of Table IX). Independent data must be used to obtain the value of $\partial \ln \gamma/\partial \ln m$. Dalton and Schmidt have collected from the literature the available values of this coefficient. They are given in Table V. In using these values it must be remembered that they are strictly valid only in the neighborhood of the temperature at which they were obtained, which here is near 0°, since they were derived from freezing point data. On the other hand none of the other data are sufficiently accurate to warrant the introduction of such a refinement as the temperature coefficient of the activity coefficient.

The values of ΔH obtained in this way by means of equation (63) are given in Table V under the heading of $(\overline{H}_{2(\text{sat'd})} - \overline{H}_{2(\text{solid})})$ calculated. These are to be compared with the values obtained from the heats of solution and dilution. It will be seen that except in the case of l-proline there is good agreement between the values obtained by these two independent methods. The agreement between the observed and calculated values of $(\overline{H}_{2(\text{sat'd})} - \overline{H}_{2(\text{solid})})$ for aspartic and glutamic acids would have been closer if a correction for the change of activity coefficient with temperature had been introduced. With these two substances the activity coefficients are large and probably are a measure of the degree of aggregation which changes markedly with temperature. Application of the temperature coefficient of the activity coefficient may possibly be justified here.

(3) Determination of the Temperature Coefficient of the Heat of Solution from Solubility Data. The temperature coefficient of the heat of solution of the solid solute into an ideal solution may be

obtained from the solubility equations of Table IX. By equation (63)

 $\frac{\partial \ln N_2}{\partial T} = \frac{\Delta H}{RT^2}$

Substituting the value of ΔH from equation (d)

$$\Delta H = RT^2(b_4 + 2C_4T)$$

The temperature coefficient of ΔH becomes

$$\frac{d(\Delta H)}{dT} = R(2b_4T + bC_4T^2) \tag{66}$$

The coefficients for calculating $d(\Delta H)/dT$ for a number of amino

 ${\bf TABLE}~{\bf X}$ Temperature Coefficients and Free Energies of Solution of Amino Acids

Amino Acid	a	b	$\frac{d(\Delta H)}{dT}$ at 298.1° (Calculated value)	C_1	C_2	$\Delta F_{298.1}$	Mole frac- tion in sat'd sol'n at 25° $N_{2(28^{\circ})}$	Activity coeffi- cient used
d-Alanine	0.0412		12	- 1750	73	- 350	0.0326	1
d.l-Alanine	0.0495		15	- 2270	91.9	- 350	0.0323	1
l-Asparagine · H ₂ O	0.468	0.00141	-14.5	12750	-122	950	0.00359	1
l-Aspartic Acid	0.139		41	- 6040	262	2550	0.000676	0.36
d.l-Aspartic Acid.	0.437	-0.00139	7	5070	30.5	2300	0.000104	0.36
l-Cystine	0.124		37	- 5520	245	4550	0.00000821	1
l-Diiodotyrosine.	0.176		52	- 7680	335	3850	0.0000259	1
d-Glutamic Acid.	0.148		44	- 6570	280	2300	0.00105	0.36
d,l-Glutamie Acid	0.139		41	- 6050	260	1750	0.00250	0.36
Glycine	0.325	0.00125	-14	7540	-107	- 650	0.0566	0.9
l-Hydroxyproline.	0.0338		10	- 1540	60.2	- 600	0.0472	1
d-Isoleucine	-0.120	0.000699	26	- 6910	171	700	0.00562	1
d,l-Isoleucine	-0.164	0.00103	42	-10740	279	1050	0.00306	1
l-Leucine	-0.151	0.000858	30	- 8100	201	950	0.00335	1.07
d,l-Leucine	-0.204	0.00126	40	- 9920	266	1500	0.00136	1.03
d,l-Methionine	0.164	-0.000342	18	- 1130	109	850	0.00406	1
d,l-Norleucine	-0.117	0.000875	23	- 4320	150	1450	0.00158	1
l-Phenylalanine	0.0636		19	- 2840	118	1000	0.00322	1
d,l-Phenylalanine	-0.0991	0.000812	42	- 9760	277	1450	0.00154	1
l-Proline	0.0301		9	- 1340	50.9	-1450	0.203	1
d,l-Serine	0.324	-0.00101	7	3320	30.2	450	0.00853	1
Taurine	0.610	-0.00240	-31	15220	- 227	100	0.0148	1
l-Tryptophane	0.193	-0.00082	-15	5830	- 99.2	1750	0.000920	1
l-Tyrosine	0.134		40	- 5970	260	3550	0.0000447	1
d-Valine	-0.0339	0.000227	10	- 2480	66	200	0.0135	0.9
d,l-Valine	-0.108	0.000707	31	- 7780	204	350	0.0107	0.9

$$\frac{d(\Delta H)}{dT} = aT + bT^2; \Delta F = -\frac{d(\Delta H)}{dT}. T \ln T + C_1 + C_2 T$$

(The data have been calculated on the basis of the solubility equations which are given in Table IX, and in the manner indicated in the text.)

acids are collected in Table X. For comparison the values for glycine and d,l-alanine calculated from the specific heat data are also given. We have discussed above some of the reasons for the differences in the two values.

(4) Change of Free Energy of Solution and of Dilution with Temperature.

In the foregoing two sections we have employed data on the change in free energy with temperature to calculate heats of reaction. We shall now do the reverse, and calculate the change with temperature of the free energy of solution and dilution from the heats of reaction.

The fundamental equation is again the van't Hoff equation (11). For physiological problems it is sufficiently accurate, if the value of ΔF and ΔH at 20° or 25° are available, to assume that ΔH is constant over the temperature range to 37°. The integrated form of equation (11), equation (12), will give values well within the limits of error of the experimental data. In all of our calculations of the free energies of substances at 37° from those at 25° we have employed this approximate equation.

(5) The General Equation for Change in the Free Energy of Solution with Temperatures. It is interesting, and of some convenience, to obtain a general equation expressing the variation of the free energy increase with the temperature, without the assumption of a constant value for ΔH . We shall derive this equation here for the reaction in which one mole of solid solute is transferred to the standard solution. We shall write this reaction

$$X_{2 \text{(solid)}} \rightarrow X_{2 \text{(aq.},a_2=1)}; \Delta H = \overline{H}_2^{\circ} - H_{2 \text{(solid)}}$$

The fundamental equations here are equations (11) and (47). Equation (47) for this reaction takes the form

$$\frac{d(\overline{H}_{2}^{\circ} - H_{2\text{(solid)}})}{dT} = \Delta C_{P} = \overline{C}_{P_{2}}^{\circ} - C_{P_{2}\text{(solid)}}$$
(67)

We shall assume that ΔC_P is a constant over the temperature range in which the final equation is to be applied. Integrating equation (67)

 $(\overline{H}_{2}^{\circ} - H_{2(\text{solid})}) = (\overline{C}_{P_{2}}^{\circ} - C_{P_{2}(\text{solid})})T + C_{1}$ (68)

The value of $(C_{P_2}^{\circ}-C_{P_2(\text{solid})})$ may be obtained directly from specific heat data as in Table VII, or the value of $d(\Delta H)/dT$ as calculated in Table X may be used. In order to obtain the value

of C_1 the value of $(\overline{H_2}^{\circ} - H_{2(\text{solid})})$ at a given temperature, T, must be known. These values at 25° are given in Table V.

In obtaining the value of ΔC_P from specific heat data, $C_{P_2}^{\circ}$ and $C_{P_2(solid)}$ must be taken at the same temperature, which can be any temperature within the range in which our assumption regarding the constancy of ΔC_P is valid.

We can now use this value of ΔH as a function of the temperature in equation (65) in the van't Hoff equation

$$d\left(\frac{\Delta F}{T}\right) = -\frac{\Delta H}{T^2} dT = -\frac{(\overline{C}_{P_2}^{\circ} - \overline{C}_{P_2(\text{solid})})T + C_1}{T^2} dT \tag{69}$$

From which

$$\int d\left(\frac{\Delta F}{T}\right) = -\int (\overline{C}_{P_2}^{\circ} - C_{P_2(\text{solid})}) d \ln T - \int \frac{C_1}{T^2} dT$$

and

$$\Delta F = -(\overline{C}_{P_2}^{\circ} - C_{P_2(\text{solid})})T \ln T + C_1 + C_2T$$
(70)

In order to obtain the value of C_2 the value of ΔF must be known at a given temperature T. We can evaluate ΔF by considering the above reaction in two stages, first, the solution of the solid into the saturated solution, and then the transfer of a mole of solute from the saturated solution to a very large volume of the standard solution in which the activity is unity. For the first stage $\Delta F = 0$ since the solid and dissolved solute are in equilibrium. ΔF for the second stage is, by equation (59) $RT \ln 0.0177/N_2$ where, at the temperature T, N_2 is the mole fraction of the solute in the saturated solutions; or, by equation (60), $RT \ln 1/m\gamma$ where m and γ are, respectively the molality and the activity coefficient of the solute in the saturated solution. We shall employ the form with the mole fraction. If activity coefficients are to be introduced we shall use the form $RT \ln 0.0177/N_2\gamma$.

Equation (70) for the whole reaction becomes

$$\Delta F = RT \ln \frac{0.0177}{N_2 \gamma} = -(\overline{C}_{P_2}^{\circ} - C_{P_2(\text{solid})})T \ln T + C_1 + C_2 T \tag{71}$$

The value of C_2 is obtained for the temperature T when the mole fraction in the saturated solution at that temperature is N_2 .

In Table XI the available data are collected for use with equation (70). For the sake of consistency we have chosen the values of $d(\Delta H)/dT$ and of $(\overline{H}_2{}^{\circ}-H_{2(\text{solid})})$ derived from the solubility data. C_1 and C_2 were computed from the ideal heats of solution and

solubilities at 25° (Tables V and IX). The values of ΔF at 25° with the mole fractions and activity coefficients used, are also given.

(6) Free Energy of Formation of the Solute in the Standard Solution. The free energy of formation of a substance in the standard solution as specified above is

$$\Delta F_{(T,a_2=1)} = \Delta F_T^{\circ} + \Delta F_{(T,\text{solution})}$$
 (72)

Values for $\Delta F^{\circ}_{208.1}$ for some of the amino acids are given in Table II and of $\Delta F_{(\text{solution 298.1°})}$ in Table X.

(7) The Free Energy of Ionization. The only remaining process to be considered in obtaining the values for the heats and free energies of amino acids in aqueous solution is their ionization. We shall adopt the now firmly established zwitterionic constitution of amino acids and formulate the ionization reactions for an hypothetical amino acid containing two acid and two basic groups as follows. We shall begin in highly acid solution and follow the ionization through to highly alkaline solution. Each reaction, whether for the acid or basic group, is the loss of a hydrogen ion

$$\begin{array}{c|c} \operatorname{Acid} & \operatorname{Neutrality} \\ R^{++} \rightarrow R^{\pm +} + H^{+}; & R^{\pm +} \rightarrow H^{+}; & R^{\pm \pm} \rightarrow R^{\pm -} + H^{+}; \\ \end{array}$$

The positions of the vertical lines indicate approximately the pH of the solutions in which the above ionizations occur.

The law of mass action expressions for these reactions are

$$\frac{[\mathbf{R}^{\pm+}][\mathbf{H}^{+}]}{[\mathbf{R}^{\pm+}]} = K_{1}; \ \frac{[\mathbf{R}^{\pm\pm}][\mathbf{H}^{+}]}{[\mathbf{R}^{\pm+}]} = K_{2}; \ \frac{[\mathbf{R}^{\pm-}][\mathbf{H}^{+}]}{[\mathbf{R}^{\pm\pm}]} = K_{3}; \ \frac{[\mathbf{R}^{=}][\mathbf{H}^{+}]}{[\mathbf{R}^{\pm-}]} = K_{4}$$

The bracketed terms in these equations represent thermodynamic activities. The constants therefore are thermodynamic constants. Nearly all of the ionization constants of amino acids in the literature were derived from expressions in which the actual molal concentrations of the two forms of the amino acid, and the hydrogen ion activity were used. These constants are called apparent ionization constants and are designated as K'. The relation between the apparent and thermodynamic ionization constants is as follows:

$$\frac{(\mathbf{R}^{-})\gamma_{-}[\mathbf{H}^{+}]}{(\mathbf{R}^{\pm})\gamma_{\pm}} = K; \quad \log K = \log \frac{(\mathbf{R}^{-})[\mathbf{H}^{+}]}{(\mathbf{R}^{\pm})} + \log \frac{\gamma_{-}}{\gamma_{\pm}} = \log K' + \log \frac{\gamma_{-}}{\gamma_{\pm}}$$

$$\therefore \quad pK = pK' - \log \frac{\gamma_{-}}{\gamma_{\pm}}$$

$$(73)$$

Free Energies of Formation, in Calories per Mole, of the Different Ionic Species of Some Amino Acids, Creatinine, Creatine, Benzoic Acid, and Histories And in Ameno Schrifton at 850 TABLE XI

	nion	$\Delta F(R^-) + 2.303 RT pK$			-154,990					-137,190			-154,030				- 67,770
	Divalent Anion (R^-)	pK			$pK_w-pK'_B, 9.47$				pK _{A2} , 10.78*	$pK_w - pK_{B_2}, 10.25$			$pK_w - pK'_{B_1} 9.47$				pK'_{A_2} , 10.15
	Anion	$\Delta F_{(N)} + 2.303 RT pK$	- 75,910	- 70,510	-167,940	- 52,650				T	•	- 82,940	-166,950	012'69 -	089,69 -	- 69,930	- 81,610
25°	Monovalent Anion (R^-)	pK	$pK_w - pK'_B, 9.69$	$pK_w - pK'_B, 9.09$ $pK_w - pK'_B, 8.9$	pK'42, 3.63	pK_A , 4.20*			$pK_w - pK_{B_1}, 8.33*$	$pK_w - pK_{B_1} = 8.00*$	$pK_w - pK'_B, 9.69$	pK_A , 3.81*	pK'_{A_2} , 4.07	$pK_w - pK'_B, 9.60$	$pK_{w}-pK'_{B_{1}}9.60$	$pK_w - pK'_B, 9.60$	$pK_w-pK'_B, 9.15$
s Solution, at	Monovalent Cation (R^+)	$\Delta F_{(N)} = 2.303 RT pK$	- 92,320	-92,920 $-186,450$	-175,480		-124,200	- 14,160	- 83,960	-164,880	- 92,720		-175,360	- 85,990	- 85,960	-86,210	- 97,130
Hippuric Acid, in Aqueous Solution, at 25°	$\begin{array}{c} \text{Monovalent} \ (R^+) \end{array}$	pK	pK4, 2.34*	$pK_A, 2.34*$ $pK'_A, 2.08$	pK'4, 1.90		pK'4, 2.67	pK'4, 4.84	pK4, 1.71*	pKA, 2.05*	pK'4, 2.31		pK'4, 2.10	pK'4, 2.34	pK'4, 2.34	pK'4, 2.34	pK'A, 2.23
Hippuric A	Divalent Cation (R^{++})	$\Delta F_{(R^+)} - 2.303 RTpK$								-166,300							
		pK								$pK_{A,1}.04*$	•						
	Neutral	$\begin{array}{c} \text{form} \\ (R_N) \\ \Delta F_{(N)} \end{array}$	- 89,130	- 89,730 -183,610	-172,890	- 58,380	-120,560	- 7,560	- 81,630	-162,080	- 89,570	- 88,140	-172,500	- 82,800	- 82,770	- 83,020	- 94,090
	Solu- tion -RTln	$M_{\text{sat'd}}$ (sol^n) $=\Delta F$ of solution	-350	-350 950	2550	2140	1250	- 10	820	4550	-650	2300	2300	950	950	1500	3550
	Crystal-	$\begin{array}{c} \text{line} \\ \text{form} \\ \Delta F_{(\text{solid})} \end{array}$	- 88,780	-89,380 $-184,560$	-175,440	-60,520	-121,810	- 7,550	- 82,480	-166,630	- 88,920	-90,440	-174,800	-83,750	-83,720	-84,520	- 97,640
		Substance	<i>d</i> -Alanine 88,780 -350	d_{sl} -Alanine - 89,380 l -Asparagine. \mathbb{H}_{s} O -184,560	l-Aspartic acid	Benzoie acid	Creatine. H.O	Creatinine	l-Cysteine	<i>I</i> -Cystine166,630	Glycine	Hippuric acid	d-Glutamic acid -174,800	l-Leucine	d-Leucine	d,l-Leucine	l-Tyrosine

* Thermodynamic ionization constants.

The values for the ionization constants of the amino acids were taken from Cohn, E. J., Ergeb. Physiol., 33, 781 (1931). The value for benzoic acid was taken from Jeffrey, G. H., and Vogel, A. I., Philos. Mag., 18, 901 (1934), and that of hippuric acid from Josephson, B. A., Biochem. Z., 267, 74 (1933).

In Table XI are values for the free energies of formation at 25° of different ionic species of a number of amino acids. In most cases only apparent ionization constants were used. The values marked with an asterisk were computed from thermodynamic ionization constants. The values for $\Delta F^{\circ}_{298.1}$ are taken from Table II, for ΔF of solution at 298.1°, from Table X. At the head of the column for each species the relation is given between its free energy and those of the parent form and the ionization constant. In the case of the cation the equation used is $\Delta F_{\text{ion}} = \Delta F_{\text{(neutral)}} - 2.303RTpK$, instead of, from equation (20), $\Delta F_{\text{(neutral)}} + 2.303RTpK$. This difference arises from the formulation of the two ionization reactions. In equation (20) it was Neutral Form+H+ \rightarrow Ion, here it is Ion \rightarrow Neutral Form+H+.

(8) Determination of the Heat of Ionization from the Temperature Coefficient of the Ionization Constant. From the ionization constants we can obtain, by means of the van't Hoff equation, values for the heat of ionization of each of the groups. Assuming that ΔH is constant over the temperature range the integrated form of this equation is equation (11).

Substituting $\Delta F = -RT \ln K$ into this equation we obtain

$$-R \ln K_2 - (-R \ln K_1) = -\Delta H \left(\frac{T_2 - T_1}{T_1 T_2} \right)$$

Therefore

$$\Delta H = -\Delta p K \left(\frac{R \times 2.303 \times T_1 T_2}{T_2 - T_1} \right) \tag{74}$$

For the temperature range 0° to 25° this becomes

$$\Delta H = -14890 \Delta p K$$

In Table XII are collected the ionization constants at 0° and 25° of the amino acids for which these data are available. The apparent ionization constants are designated as pK', the thermodynamic constants as pK.

The figures show that in monoamino monocarboxylic acids the heat of ionization of the group whose degree of ionization changes in acid reaction is small compared with that of the group which ionizes in alkaline reaction. In other words d(pK)/dT for the ionizable group in acid reaction is small compared with that of the group in alkaline reaction. This is one of the strong pieces of evidence in favour of the zwitterionic constitution of amino acids,

Table XII

Heats of Ionization of Amino Acids Calculated from the Temperature Coefficients of the Ionization Constants.

		Ionizati stan	on Con- ts at		ΔH	Probable group to
Amino Acid		0° pK _w =14.94	25° pK_{w} $= 14.00$	$-\Delta pK$	(calo- ries)	which the constant refers
Alanine	pK_1' pK_2'	2.31 10.47	$2.34 \\ 9.69$	$-0.03 \\ 0.78$	-450 11,610	carboxyl amino
Arginine	pK_1' pK_2' pK_3'	2.24 9.79 13.31	2.17 9.04 12.48	0.07 0.75 0.83	1050 11,150 12,350	carboxyl amino guanidine
Aspartic acid	pK_1' pK_2' pK_3'	2.01 3.77 10.31	1.90 3.63 9.47	0.11 0.14 0.84	$1650 \\ 2100 \\ 12,500$	1st carboxyl 2nd carboxyl amino
α-Amino- n-butyric acid	pK_1' pK_2'	2.51 10.44	2.55 9.60	-0.05 0.84	-750 12 ,500	carboxyl amino
β-Hydroxy-α- amino- <i>n</i> -butyric acid	pK_1' pK_2'	2.63 10.43	2.71 9.62	-0.08 0.81	-1200 $12,500$	carboxyl amino
Diiodotyrosine	pK_1' pK_2' pK_3'	2.21 6.53 8.31	2.12 6.48 7.82	0.09 0.05 0.49	1350 750 7330	carboxyl phenolic amino
Glutamic acid	pK_1' pK_2' pK_3'	2.23 4.14 10.22	2.10 4.07 9.47	0.13 0.07 0.75	1950 1050 11,150	1st carboxyl 2nd carboxyl amino
Histidine	pK_1' pK_2' pK_3'	1.90 6.50 9.75	1.82 6.04 9.12	0.08 0.46 0.63	1200 6850 9370	carboxyl imino amino
Lysine	pK_1' pK_2' pK_3'	2.20 9.81 11.31	2.18 8.95 10.53	0.02 0.86 0.78	300 12,800 11,600	carboxyl 1st amino 2nd amino
Valine	$pK_1' \\ pK_2'$	2.32 10.33	2.32 9.62	0 0.71	0 10,570	carboxyl amino

(The data were taken from Cohn, E. J., Ergeb. Physiol., 33, 781 (1931), and recalculated as indicated in the text). See also Jukes, T. H., and Branch, G. E. K., Science, 80, 228 (1934).

and of assigning the ionization in acid solution to the carboxyl group and in alkaline solution to the amino group. The ionization of the latter group includes within it the ionization of water. Hence the values for this group are much higher than for the carboxyl group. It also varies much more with temperature because the ionization constant of water varies markedly with temperature, whereas that of weak organic acids shows very little temperature variation.

Table XIII gives the thermodynamic ionization constants of d,l-alanine at different temperatures obtained by Nims and Smith

Table XIII

Thermodynamic Ionization Constants of d,l-Alanine and Heats of Ionization at Different Temperatures

Temperature (degrees)	pK_1	ΔH (calories)	pK_2	ΔH (calories)	
20	2.350	913	10.006	10900	
25	2.340	720	9.870	10810	
30	2.332	538	9.740	10740	
35	2.327	369	9.615	10680	
40	2.324	215	9.494	10630	
45	2.322	79	9.378	10580	

(Nims, L. F., and Smith, P. K., J. Biol. Chem., 101, 401 (1933).

(6). ΔH was not assumed constant as in Table XI, but was obtained at each temperature from the slope of the curves of log K plotted against T. These figures for ΔH at 25° compared with those for alanine in Table XI show the order of magnitude of the error incurred when apparent ionization constants are used and ΔH is assumed to be constant.

6. FREE ENERGY CONSIDERATIONS OF SOME BIOCHEMICAL PROCESSES

(1) The Synthesis of Urea. We shall proceed to apply the free energy data which have been obtained to some physiological and biochemical questions. We shall consider first the synthesis of urea from ammonia and carbon dioxide under the conditions in which these substances are normally found in the plasma. This reaction has been chosen although it is not one in which amino acids are involved because it illustrates clearly a new point of view which the application of the second law of thermodynamics to physiological questions has introduced.

We may write two mechanisms for the synthesis of urea:

- (1) $2NH_3+H_2CO_3$ (aq.) $\rightarrow CO(NH_2)_2+2H_2O$; and
- (2) $2NH_4^+ + 2HCO_3^-$ (aq.) $\rightarrow CO(NH_2)_2 + 2H_2O + H_2CO_3$ (aq.)

The heat contents and free energies of the participants in these two reactions are given in Table XIV.

Table XIV

Heat Contents ΔH , and Free Energies of Formation ΔF , of Substances Entering into the Synthesis of Urea

	$egin{array}{l} ext{Heat} \ ext{Content} \ ext{ΔH} \end{array}$	Free Er in Standard State at 25°	nergy of Format in Standard State at 37.4°	ion, ΔF in Plasma at 37.4°	
NH ₃ (aq.)	$\begin{array}{r} -31,790 \\ -167,470 \\ -164,000 \\ -68,310 \\ -79,870 \end{array}$	calories - 6,300 - 18,930 - 148,810 - 140,000 - 56,720 - 47,410 - 49,010	calories - 5,790 - 18,440 -148,000 -139,010 - 56,230 - 46,060 - 47,820	calories - 12,580 - 23,090 - 152,180 - 141,670 - 56,230 - 51,080	

(Unpublished calculations based on data which are given by Lewis and Randall, and by Parks and Huffman. See reference section.)

The values of ΔH° and ΔF° are taken from Table II. The free energies at 37.4° were computed by means of equation (12).

The standard free energy of urea (aqueous) at 37.4° was computed from the free energy of the solid at this temperature and a mole fraction of the saturated aqueous solution of 0.308. The concentration in the plasma was taken as 0.005 molal.

The free energy of the bicarbonate ion at 37.4° was computed from the free energy of $H_2CO_{3(aqueous)}$ at this temperature and the value of $10^{-6.33}$ for the dissociation constant of the first hydrogen. $\Delta F_{(HCO_3,\ 310.4)}$ from equation (20) is $-148,000+(1.9867\times310.4\times2.303\times6.33)=-139,010$ calories. A similar calculation gave the free energy of the bicarbonate ion at 25°. pK_1 at this temperature is 6.34. From the free energies of the bicarbonate ion at these two temperatures ΔH was computed.

In the estimation of the free energies of H₂CO₃ and HCO₃ in plasma the pH was taken as 7.4; and the CO₂ tension as 0.02 atmospheres; the concentration of the bicarbonate ion was obtained from the equation

$$pH = pK' + \log \frac{(\text{HCO}_3^-)}{\frac{P}{760} \alpha_{\text{CO}_2} \cdot \frac{1000}{22400}}$$
 (75)

where pK' is the apparent dissociation constant at 37.4°, P the partial pressure of $CO_2 = 0.05(760-48)$, and α the solubility of CO_2 in serum (0.54 at 38°). For pK' we chose 6.15; from the equation of Hastings and Sendroy (7) $pK' = 6.33 - 0.5\sqrt{\mu}$, where μ , the ionic strength of blood, is taken as 0.13. The molal concentration of H_2CO_3 was estimated in this manner to be 1.13×10^{-3} , and of HCO_3 as 1.995×10^{-2} . Allowance was made for the activity coefficient of the bicarbonate ion by calculating its free energy in plasma from that of the undissociated molecule in plasma by means of the equation

$$\text{H}_2\text{CO}_{3(\text{aq.1.13}\times10^{-3}\text{M})} \rightarrow \text{HCO}_{3^{-}(\text{aq.1.995}\times10^{-2}\text{M})} + \text{H}_{(a=10^{-7.4}\text{M})}; \Delta F = 0.$$

From which

$$\begin{split} \Delta F_{\text{(HCO}_3^-,310.4^\circ,1.995\times10^{-2}\text{M})} = & \Delta F_{\text{(H}_2\text{CO}_3\text{aq.,310.4}^\circ,1.13\times10^{-8}\text{M})} - \Delta F_{\text{(H}^+,310.4^\circ,10^{-7.4}\text{M})} \\ = & -152,180 - (RT \text{ ln } 10^{-7.4}) \\ = & -141,670 \text{ calories.} \end{split}$$

If we had calculated the free energy of the ion in plasma from its molality without allowing for an activity coefficient, i.e., by adding $RT \ln 1.995 \times 10^{-2}$ to the free energy of the ion in its standard solution at 37.4°, the value would have been -141,420 calories. The difference between these two values represents an activity coefficient correction.

From the free energies of aqueous ammonia and ammonium ion at 37.4° the dissociation constant of ammonia was computed by means of the equation

$$NH_{3(1M)} + H^{+}_{(1M)} \rightarrow NH_{4}^{+}_{(1M)}; \quad \Delta F = -18,440 + 5790$$

= -12 650 calories

From which

$$RT \ln K = 12,650; K = 8.1 \times 10^8$$

At pH 7.4 therefore, 97 per cent of the total ammonia is in the form of the ammonium ion. We have assumed that all of the ammonia is ionized and have taken its concentration in plasma as 0.00055 molal, which led to the free energy of the ammonium ion in plasma at 37.4°.

The mole fraction of the water in the blood was taken as 1.

With these data we shall calculate ΔH for each of the mechanisms postulated. The concentrations of the reactants and products may be ignored. The heats of dilution are small and will very nearly cancel out. According to mechanism (1) $\Delta H = (-76,270) + 2(-68,310) - 2(-19,160) - (-167,470) = -7100$ calories. According to mechanism (2) $\Delta H = (-76,270) + 2(-68,310) + (-167,470) - 2(-31,790) - 2(-164,000) = 11,220$ calories. The difference in the two values of ΔH is the heat of ionization. Reaction (1) is exothermic, reaction (2) is endothermic. From the values of ΔH alone it is not safe even to guess whether the synthesis of urea from ammonia and carbon dioxide in the liver can proceed spontaneously or not, i.e. whether it is dependent on energy liberating reactions or not.

There is no uncertainty when the free energy data are considered. Since the ions and the undissociated molecules are in equilibrium ΔF will be the same regardless of the mechanisms postulated for the reaction. Thus from reaction (1) ΔF under the conditions in the plasma is (-51,080)+2(-56,230)-2(-12,580)-(-152,180)=13,800 calories; and from reaction (2) (-51,080)+2(-56,230)+(-152,180)-(-23,090)-2(-141,670)=13,800 calories.

The synthesis of urea from ammonia and carbon dioxide under these conditions results in a gain in free energy for the system as formulated above. This formulation is therefore incomplete; because this reaction cannot take place spontaneously. The free energy gained, as it were, by the urea must be supplied by another free energy yielding reaction in such quantity that ΔF for the complete reaction *i.e.*, the reaction that liberates energy plus the reaction that absorbs energy, is negative.

The conditions under which the synthesis of urea from ammonia and carbon dioxide occurs in the liver are sufficiently similar to those postulated for the plasma for the same general conclusion to hold.

(2) Anabolism and Catabolism from the Point of View of the Free Energy Change. From the physiological point of view urea formation is a catabolic phenomenon; from the physiochemical point of view this "half-reaction" is an anabolic process because there is a gain in free energy which must be supplied by some other energy-yielding reaction. This complete antithesis between physiological and physicochemical classification raises the question whether it is profitable any longer to retain the classical concepts

of anabolism and catabolism. As far as physiological chemistry is concerned there can be no question that it will be more profitable to ahandon them. The alternative is to classify reactions according to whether they can proceed spontaneously, i.e. ΔF is negative; or whether they require the driving force of another reaction, i.e. the reaction as formulated is only a "half-reaction" in which ΔF is positive. The value of this alternative classification is that, in the case of a reaction in which ΔF is positive, it immediately points to the existence of another linked reaction. This calls up a number of other questions regarding the mechanisms whereby the energy is transferred, the "efficiency" of the complete reaction, i.e. the ratio of the energy stored to that produced by the energy yielding reaction. In the case of the synthesis of urea, the ornithine-citrullinearginine cycle for the synthesis of urea proposed by Krebs is clearly only part of the reaction. This cycle cannot proceed spontaneously. It must be driven. We possess at present no information regarding the nature of this driving mechanism. There are only a few observations which may throw some light on this question. Under some conditions there is an increased oxygen consumption when urea is synthesized from ammonia and carbon dioxide by the liver, under other conditions there is not. The rate of synthesis is faster in a medium containing high concentrations of lactate than when glucose is provided. The synthesis requires intact cell structure, and is suspended by lowering the temperature to 25° or by anaerobiosis.

(3) Synthesis of Aspartic Acid (8). We shall consider now two different types of synthesis of amino acids, both of which have been observed to occur in surviving animal liver.

When fumaric acid and ammonia are incubated with surviving liver or added to a perfusing fluid, *l*-aspartic acid is synthesized. The equilibrium between these three substances at pH 7.4 and 37° has been measured with toluene treated *Bacillus coli communis* as catalyst. The equilibrium concentrations in one experiment were

$$NH_4^+$$
 (0.028 M)+Fumarate= (0.028 M) \rightleftharpoons l-Aspartate- (0.072 M).

Inserting appropriate activity coefficients the equilibrium expression is

$$\frac{(0.072)(0.7)}{(0.028)(0.7)(0.028)(0.22)} = 417$$

from which we may write

Fumarate= $_{(1M)}$ +NH₄+ $_{(1M)}$ $\rightarrow l$ -Aspartate= $_{(1M)}$; $\Delta F_{310} = -3720$ calories

Sufficient thermal data are available for the calculation of this free energy change. These are collected in Table XV. The following data not given in the table were used in obtaining these values. The solubility of fumaric acid at 37° is 0.0827 moles per 1000 grams of water. In the saturated solution it is 3 per cent ionized. The ionization constants at 37° are pK_1 3.04, pK_2 4.51. The free energy of solution of l-aspartic acid at 37° was calculated from the data in Table X. The ionization constant used was pK_{A_2} 3.57. The free energies of the neutral molecules at 37° were calculated from those at 25° by means of equation (12) with the values of ΔH given in the table. The free energy of the NH₄+ ion was calculated for 37° from the data given in Table XIV by means of equation (12).

Table XV

Heat Contents, ΔH , and Free Energies of Formation, ΔF , of Fumaric Acid, l-Aspartic Acid and Ammonium Ion at 37°

Substance	ΔH	$\Delta F^{\circ}_{298.1}$	$\Delta F^{\circ}_{310,1}$	ΔF for dilution	ΔF for ioniza-	$\Delta F_{310.1}$ of ion
Fumaric Acid l-Aspartic acid. NH ₄ +		calories -157,230 -175,440	calories -155,710 -173,110	calories 1560 2270	calories 10,720 5,070	calories -143,430 -165,760 - 18,410

The agreement between the thermal and equilibrium values for the free energy change is well within the experimental error of either value.

From the equilibrium constant for this reaction we may state that a significant amount of aspartic acid can be synthesized from ammonia and fumarate under physiological conditions without the driving force of any other reaction. Thus 1 mg. per cent of ammonia and 116 mg. per cent of fumarate ions are in equilibrium with 8 mg. per cent of aspartic acid.

(4) Synthesis of Alanine. The synthesis of alanine from pyruvic acid and ammonia has also been observed to take place in the liver. The reverse reaction, the oxidative deamination of alanine to form pyruvic acid and ammonia, has been observed in the kidney

and in the intestinal mucosa. We shall calculate the free energy change at 25° from thermal data. The equilibrium is too far over on the side of pyruvic acid and ammonia for direct measurement of the equilibrium constant.

We shall write the reaction

All substances are in aqueous solution at unit activity. The free energy of the pyruvate ion at 25° has been estimated by Wurmser and Mayer-Reich (9) as -109,670 calories. This value is derived from an equilibrium constant which they measured between lactate and pyruvate and is based upon an assumed value for lactate. The possible error for our present purpose is negligible. The other free energy data are given in Tables XI and XIV.

For Pyruvate⁻(1 M) + NH₄⁺(1 M) \rightarrow d-Alanine[±](1 M) + $\frac{1}{2}$ O₂ (1 atmos.); $\Delta F_{298.1} = (-89,130) - (-109,670) - (-18,930) = 39,470$ calories.

From which value the equilibrium constant for the reaction as written is, by equation (10) $10^{-28.4}$. The equilibrium is so far over on the side of deamination that we can be certain that the synthesis of alanine from pyruvic acid and ammonia which has been observed must have occurred in conjunction with another energy-yielding reaction.

(5) Synthesis of Leucylglycine. We shall now consider the synthesis of two types of peptide bond. The first will be that of d,l-leucylglycine from d,l-leucine and glycine. Since at the pH of the tissues all the participants will be in the neutral form we can ignore their ionization constants. We have no data at present for the solubility of the peptide. We shall therefore calculate the equilibrium constant from the values of $\Delta F^{\circ}_{298.1}$, i.e. from those of the solids. It is extremely unlikely that the value of ΔF in solution will, for our purposes, be significantly different. The reaction is

Glycine (solid)+
$$d$$
, l -Leucine (solid) $\rightarrow d$, l -Leucylglycine (solid)
+ H_2O (liquid); $\Delta F_2 = (-113, 790) + (-56,720) - (-88,920)$
- $(-84,520) = 2930$ calories

From which K, the equilibrium constant, is $10^{-2.15}$. The equilibrium of the reaction lies so far over on the side of hydrolysis, that it is practically certain that the synthesis of the peptide bond from

amino acids of this type in vivo can occur only when it is associated with another energy yielding reaction.

(6) Synthesis of Hippuric Acid. In Table XI are the values of $\Delta F_{298.1}$ in their standard aqueous solutions of glycine, benzoic acid, and hippuric acid. At the pH of the tissues the reaction is

Benzoate⁻(aq)+Glycine[±](aq)→Hippurate⁻(aq)+H₂O (liquid) $\Delta F_{298,1}$ = (-82,940)+(-56,720)-(-52,650)-(-89,570) = 2560 calories From which the equilibrium constant, K, is 0.0133.

From this value of the equilibrium constant, hippuric acid when present at an initial concentration of 0.1 molal would be 97 per cent hydrolyzed. At concentrations of 0.01 molal or less, more than 99 per cent would be hydrolyzed at equilibrium at 25°.

The synthesis of hippuric acid is known to proceed rapidly in vivo even though the concentrations of benzoic acid and glycine are relatively very low. In the authors' laboratory, using surviving liver slices, about 40 per cent of the benzoic acid was found to be converted to hippuric acid, when the initial concentration of benzoic acid was 0.0025 M and of glycine 0.01 M.

It follows therefore that the synthesis of hippuric acid in vivo does not proceed "under its own steam," as it were, i.e. simply as a consequence of increasing the concentration of benzoic acid and glycine. The "steam," e.g. the driving force, the free energy, must be supplied by some other reaction in which sufficient energy is liberated to supply that bound up as hippuric acid and some excess, because ΔF for the complete reaction must be negative.

(7) "Equilibrium" or "Steady State" in the Tissues? Determinations of the free energy of formation of the peptide bond in other peptides gave values of the same order of magnitude as those of d,l-leucylglycine and of hippuric acid. These few free energy values are sufficient to call for a revision of the common view that a sensitive "equilibrium" exists in the tissues between protein, amino acids, and ammonia. This idea is usually expressed in the form Protein amino acids ammonia. A fasting salmon swimming up stream to the spawning grounds loses 35 per cent of its muscle protein. Following the feeding of protein to a starved frog the weight of the liver is doubled in a short time. Yet the concentration of free amino and non-protein nitrogen in the tissues varies very little. A sensitive dynamic "steady state" is maintained between protein and amino acids; as it is analogously in the case of glycogen and its derivatives. The important point

which the above free energy values bring out is that this "steady state" is not moved to the "right" or "left" by a mere addition per se of amino acids to the tissues. This may be a contributory factor, but more important is the continually applied driving force of energy-yielding reactions. These maintain the "steady state" and determine its position. When they are suspended, or when the mechanism of energy transfer is interfered with, autolysis sets in.

We are thus led to a conception of the organism as a chemical system which is more pointed and informative in its suggestion of important intracellular mechanisms than the conventional anthropomorphic concepts, such as anabolism and catabolism, endogenous and exogenous metabolism. These concepts originated in the discussion of nitrogen metabolism in animals at the end of the nineteenth and the beginning of the twentieth centuries and are based on the dubious analogy of the organism to a machine. At that time it was concluded that urea formation in vivo is an hydrolytic phenomenon with the evolution of only a small amount of energy; because it is a catabolic process, and the formation of urea through the agency of oxidative processes would be wasteful. The same line of argument led to the conclusion that deamination is a hydrolytic process. Recent work has demonstrated that both of these conclusions are incorrect. These examples show the determining influence of a general theory on the nature of the views held regarding specific physiological chemical processes and on the lines of investigation pursued. The advantage of the alternative point of view we have suggested is that it is derived solely from experimental data; and indicates clearly new lines of investigation from our present position.

(8) Synthesis of Giant Chromosomes. The recent discovery of the structure of the giant chromosomes in the salivary glands of Drosophila larvae has drawn the attention of geneticists to the question of the synthesis of genes, of nucleoproteins, of nucleic acids within the nucleus. The structure of these giant chromosomes is simply a manifold repetition of the chromosomes in other cells. The mechanism which has been postulated for the growth of these chromosomes is that purines and amino acids are "adsorbed" along the parent chromosomes in specific loci. The contours of these loci contain molecular configurations similar to or identical with those of the adsorbed molecules—and so the repetition of the identical pattern. At these loci, as a consequence of their propinquity to the necessary enzymes which are postulated, the

synthesis of the adsorbed molecules into nucleic acids and proteins occurs.

The mechanism cannot be as simple as this. Energy must be provided for the synthesis at the right time and place. Mechanisms for the production of the energy and its transfer must be included in the picture. It is possible that the synthesis of a peptide bond. for example, is not the result of the direct union of a free amino and a free carboxyl group, but of two quite different radicals. The nature of these radicals would be such that ΔF for their interaction is negative and hence their union can occur spontaneously. This mechanism is chemically and energetically much simpler, as far as the actual mechanism of synthesis is concerned, than that which has been postulated for the repetition of pattern in the giant chromosomes. But it is incompatible with the hypothesis that the repetition of pattern is achieved by the adsorption of molecules whose configuration is identical with that of the locus at which they are adsorbed. The identity, according to the above alternative mechanism, appears after the synthesis, but not before. Some other mechanism than identity of molecular configuration would then be necessary for the aggregation of a specific pattern of molecules in a given locus.

(9) The Creatine-Creatinine Equilibrium. Data are available for the calculation of the free energy change in the reaction

From the data in Table XI, $\Delta F_{298.1}$ for the reaction between neutral molecules is (-7560) + (-56,720) - (-63,840) = -440 calories.

Edgar and Shiver (10) have obtained a value for the equilibrium constant by measuring the independent reaction rate constants of Creatine aq. \rightarrow Creatinine +H₂O, and of the reverse reaction. They obtained the expression:

$$\log K = \frac{-1084}{T} + 3.3652$$

At 25° log K = -0.27, from which ΔF is -370 calories. The thermal and direct experimental values of the free energy change, and therefore of the equilibrium constant, are practically identical.

(10) The Cystine-Cysteine Oxidation-Reduction Potential. The cystine-cysteine oxidation-reduction potential is a case where the thermal and direct experimental values differ by much more than the possible experimental errors in either measurement. It also

furnishes us with an example of the use of free energy data in computing electrode potentials. In this connection see also Borsook and Schott (11).

The electrode equation for this system is

$$\begin{split} E_{\rm obs} = & \tilde{E} - \frac{RT}{2f} \ln \frac{({\rm RSH})^2}{({\rm RSSR})} - \frac{RT}{2f} \ln \frac{(K_1^{\rm SH})^2}{K_1^{\rm SS} K_2^{\rm SS}} \\ & [{\rm H}^+]^4 + K_1^{\rm SS} [{\rm H}^+]^3 + K_1^{\rm SS} K_2^{\rm SS} [{\rm H}^+]^2 \end{split}$$

$$-\frac{RT}{2f} \ln \frac{+K_1 \text{SS} K_2 \text{SS} K_3 \text{SS} [\text{H}^+] + K_1 \text{SS} K_2 \text{SS} K_3 \text{SS} K_4 \text{SS}}{([\text{H}^+]^3 + K_1 \text{SH} [\text{H}^+]^2 + K_1 \text{SH} K_2 \text{SH} [\text{H}^+] + K_1 \text{SH} K_2 \text{SH} K_3 \text{SH})^2}. (76)$$

 \tilde{E} is the so-called molal electrode potential; (RSH) and (RSSR) are the concentrations of total cysteine and cystine respectively; K_1^{SH} , K_2^{SH} and K_3^{SH} are the ionization constants of cysteine, and $K_1^{\text{SS}} \cdots K_4^{\text{SS}}$ of cystine; [H+] is the hydrogen ion activity.

E is calculated from thermal data. It is derived from the difference in free energy of cystine and cysteine when each is taken at unit activity. From the data in Table XI, the reaction is written

RSSR_(aq.,1M.,298.1°)+2H⁺_(aq.,1M.,298.1°)+2
$$e$$
 \rightarrow 2RSH_(aq.,1M.,298.1°);
 $\Delta F = -1180$ calories

From the relation $\Delta F = -Enf$, the molal electrode potential \tilde{E} is

$$\frac{1180}{2 \times 23074} = 0.025 \text{ volts}$$

With this value of the characteristic potential we can compute the potential for all concentrations of cysteine and cystine at any hydrogen concentration. The values of the ionization constants are given in Table XI. For such concentrations of total cysteine and cystine that the term $\log (RSH)^2/(RSSR)$ is equal to zero, the thermal value for $E_{obs.}$ at 25° and at pH 7.0 is -0.390 volts. Three experimental values have been reported, +0.13, -0.22 volts, and -0.33 volts.

We cannot present here a detailed discussion of these discrepancies. In the introduction we have given the reasons, which in general lead us to accept the thermal values as the final reference values. In all three of the the experimental determinations of the potential of the cystine—cysteine system there is experimental evidence that important reactions were not taken into account in the formulation of the experimental reaction.

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CHAPTER XVI

DIPOLAR IONIC STRUCTURE AND SOLUBILITY OF AMINO ACIDS, PEPTIDES, AND PROTEINS*

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INTRODUCTION

Certain general relationships between solubility and chemical structure have long been recognized. The presence in a molecule of certain polar groups, such as -OH, -COOH, and $-NH_2$, tends to make it soluble in water; the presence of hydrocarbon radicals tends to decrease solubility in water and increase it in organic solvents. Thus in homologous series such as the alcohols or fatty acids, the lower members show a strong affinity for water as compared with organic solvents; the higher members are nearly insoluble in water, while they dissolve readily in ether, benzene or carbon tetrachloride.

The properties of substances in the solid state are intimately related to solubility. Of two solids which form ideal solutions in a given solvent, the one having the higher melting point will be less soluble (1), and its solubility will increase with temperature more rapidly than that of the other. Even in non-ideal solutions the same rule frequently applies, if we consider two solutes closely related in chemical structure.

All these general rules are applicable to the amino acids as to other organic compounds. Often, indeed, they can be stated in quantitative form, as will be indicated in the course of this chapter. The structure of amino acids and peptides, however, possesses one feature of fundamental importance which sets them apart from other organic molecules. Even in the neutral, isoelectric condition these molecules bear electric charges; they are dipolar ions, or zwitterions. The evidence is strong that the same is true of pro-

^{*}The subject matter of this chapter will be more extensively and systematically treated in a forthcoming monograph on dipolar ions by Edwin J. Cohn and John T. Edsall, to be published in the American Chemical Society Monograph Series.

¹ Some of the evidence for this statement is given in Chapter XI, which should be consulted on this point in connection with the further analysis given here.

teins and phospholipids. The predominant structure of an amino acid should then be represented by the formula $^+H_3N \cdot R \cdot COO^-$ rather than the formula $H_2N \cdot R \cdot COOH$.

This dipolar ionic structure influences profoundly the physicochemical characteristics of the amino acids, and many of their chemical reactions. In particular, it is a factor of the first magnitude in determining their solubility behavior. The first part of this chapter will, therefore, be devoted to a discussion of the characteristics of dipolar ions.

SECTION I. AMINO ACIDS, PEPTIDES, AND PROTEINS AS DIPOLAR IONS

1. The Equilibrium Between Dipolar Ions and Uncharged Molecules

A simple amino acid may exist in the two tautomeric forms, $^{+}\text{H}_{3}\text{N}\cdot\text{R}\cdot\text{COO}^{-}$ and $\text{H}_{2}\text{N}\cdot\text{R}\cdot\text{COOH}$. Since the physical and chemical properties of the two are very different, it is important to know the relative numbers of two forms present in solution. This ratio will be denoted by K_{z} .

$$K_Z = \frac{(^{+}\text{H}_3\text{N} \cdot \text{R} \cdot \text{COO}^{-})}{(\text{H}_2\text{N} \cdot \text{R} \cdot \text{COOH})}$$
(1)²

Perhaps the most satisfactory method of evaluating this constant is one due to Ebert (2, 3, 4). It depends on the relative dissociation constants of amino acids and their esters. The cation of the amino acid may give off a hydrogen ion from either the carboxyl or the charged amino group; two dissociation constants are therefore involved.

$$\frac{(\mathrm{H}^{+})(^{+}\mathrm{H}_{3}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})}{(^{+}\mathrm{H}_{2}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})} = K_{A} \quad (2) \qquad \frac{(\mathrm{H}^{+})(\mathrm{H}_{2}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})}{(^{+}\mathrm{H}_{3}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})} = K_{B} \quad (3)$$

Each of the two neutral isoelectric forms may give off a hydrogen ion to form the amino acid anion.

$$\frac{(\mathrm{H}^{+})(\mathrm{H}_{2}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COO}^{-})}{(^{+}\mathrm{H}_{3}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COO}^{-})} = K_{C} \qquad (4) \qquad \frac{(\mathrm{H}^{+})(\mathrm{H}_{2}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COO}^{-})}{(\mathrm{H}_{2}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})} = K_{D} \qquad (5)$$

² This and the following equations are written in terms of the activities of the components, but in dilute solutions these activities may be taken as virtually equal to the concentrations.

The ratio of dipolar ions to uncharged molecules is, in terms of these constants,

$$K_Z = \frac{(^{+}\text{H}_3\text{N} \cdot \text{R} \cdot \text{COO}^{-})}{(\text{H}_2\text{N} \cdot \text{R} \cdot \text{COOH})} = \frac{K_A}{K_B} = \frac{K_D}{K_C}$$
(6)

The dissociation constants, determined directly by titration, are related to the constants given above, as follows:

$$K_{1} = \frac{(\mathrm{H}^{+})\left[(^{+}\mathrm{H}_{3}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COO}^{-}) + (\mathrm{H}_{2}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})\right]}{(^{+}\mathrm{H}_{3}\mathrm{N}\cdot\mathrm{R}\cdot\mathrm{COOH})} = K_{A} + K_{B} \qquad (7)$$

$$K_{2} = \frac{(\mathrm{H}^{+})(\mathrm{NH}_{2} \cdot \mathrm{R} \cdot \mathrm{COO}^{-})}{[(^{+}\mathrm{H}_{2}\mathrm{N} \cdot \mathrm{R} \cdot \mathrm{COO}^{-}) + (\mathrm{H}_{2}\mathrm{N} \cdot \mathrm{R} \cdot \mathrm{COOH})]}; \quad \frac{1}{K_{2}} = \frac{1}{K_{c}} + \frac{1}{K_{D}}$$
(8)

 K_1 and K_2 can be determined by direct experiment. If, in addition, any one of the four constants, K_A , K_B , K_C , or K_D , is known, the values of the other three are uniquely determined by equations (6), (7), and (8).

Of these constants, K_B can be determined as the dissociation constant of the ester of the amino acid in question, if it is assumed that the $-\text{COOCH}_3$ or $-\text{COOC}_2\text{H}_5$ group in its effect on the dissociation of a neighboring group (5).

$$K_{B} = \frac{\begin{pmatrix} \mathbf{R} & \mathbf{NH_{2}} \\ \mathbf{COOH} \end{pmatrix} (\mathbf{H}^{+})}{\begin{pmatrix} \mathbf{R} & \mathbf{NH_{2}} \\ \mathbf{R} & \mathbf{NH_{3}^{+}} \\ \mathbf{R} & \mathbf{NH_{3}^{+}} \end{pmatrix}} = \frac{\begin{pmatrix} \mathbf{NH_{2}} \\ \mathbf{R} & \mathbf{NH_{3}^{+}} \\ \mathbf{R} & \mathbf{NH_{3}^{+}} \end{pmatrix}}{\begin{pmatrix} \mathbf{R} & \mathbf{NH_{3}^{+}} \\ \mathbf{R} & \mathbf{NH_{3}^{+}} \end{pmatrix}} = K_{E}$$
(9)

Equation (9) is only a good approximation, whereas the earlier equations are exact in sufficiently dilute solutions. Assuming $K_B = K_E$, the following relations hold:

$$K_A = K_1 - K_E$$
 (10) $K_Z = \frac{K_A}{K_E} = \frac{K_A}{K_E} = \frac{K_1}{K_E} - 1$ (11)

If K_1 is very much larger than K_E , which proves to be the case for all the aliphatic amino acids, we have, as a close approximation

$$K_Z = K_1/K_E$$
 (12) and $\log K_Z = pK_E - pK_1$ (12a)

The application of this method to some simple amino acids and one peptide has yielded the results which are given in Table I (3).

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Substance	pK_1	pK_2	$p\mathrm{K}_{\mathrm{Ester}}$	$\begin{vmatrix} \log K_Z \\ = pK_E - pK_1 \end{vmatrix}$	K_{Z}
Glycine	2.31	9.72	7.73	5.42	260,000
α-Alanine	2.39	9.72	7.80	5.41	260,000
α-Amino-n-butyric acid.	2.55	9.60	7.71	5.16	145,000
Leucine	2.34	9.64	7.63	5.29	195,000
β-Alanine	3.60	10.19	9.13	5.53	340,000
e-Aminocaproic acid	4.43	10.75	10.37	5.94	870,000
Glycyl-glycine	3.14	8.07	7.75	4.61	41,000

(All values are at 25°.)

(Edsall, J. T., and Blanchard, M. H., J. Amer. Chem. Soc., 55, 2337 (1933).)

Too much stress should not be laid on the exact values obtained, but it is clear that the dipolar ion overwhelmingly predominates over the uncharged molecule in all these substances, the latter being negligible in aqueous solution.

(1) Influence of Multiple Charges. In an amino acid containing more than two dissociating groups, several isoelectric forms are possible. Thus aspartic acid may exist in three different forms, each having a net charge of zero:

Calculation shows that the uncharged molecule (C) exists in negligible amounts, the dipolar ion with charges close together (A) being the predominant form (3).

Likewise an isoelectric dibasic amino acid, such as lysine, may exist in the three forms:

Here again, the concentration of the uncharged molecule (C) is negligible, but in this case the form (B), with wide separation between the charged groups, predominates (3). This is important because the two forms differ widely in their effect on the dielectric constant of the solution (see below) and thereby influence the activity of all substances in the solution.

As the number of dissociating groups in the molecule increases, the number of possible isoelectric forms will increase rapidly. In an isoelectric protein the number of possible forms will be very large, and several different forms may contribute appreciably to the actual state of the protein in solution.

(2) Influence of Temperature and Solvent. By studying the dissociation of glycine and its methyl ester at several temperatures, the change of K_z with temperature has been estimated for glycine from equation (12a). K_z is thus found to be approximately 310,000 at 20°, and 160,000 at 30°, and it should become still lower at higher temperatures (3).

The dissociation constants of amino acids and their esters have been studied in alcohol water-mixtures (3, 6). As the proportion of alcohol in the solvent increases, the shift of the dissociation constants indicates a steady fall in the value of K_z . In absolute alcohol, dipolar ions and uncharged molecules may be present in proportions not far from equality, although the dipolar ions probably still predominate.

2. Spectroscopic Evidence for the Existence of Dipolar Ions

(1) Raman Spectra. If a beam of monochromatic light (of frequency ν_0) traverses a clear liquid, free of dust particles, most of the light passes through in the direction of the beam, but a small amount is diffused in other directions by the action of the molecules in the liquid. The spectrum of this faint diffused light (generally observed at right angles to the original beam) when photographed contains, in addition to the line of frequency ν_0 , fainter lines of frequency, ν_1 , ν_2 , ν_3 , etc. The frequency shifts, $\Delta\nu_1=\nu_0-\nu_1$, $\Delta\nu_2=\nu_0-\nu_2$, are independent of the particular frequency, ν_0 , used to produce the spectrum, and characteristic of the substances present in the liquid. They represent characteristic frequencies of molecular vibration and yield important information about the forces binding the atoms in the molecule. This effect, discovered by Raman in 1928, has already been studied in more than fifteen hundred different molecules (7, 8).

The Raman spectra of some amino acids, fatty acids, and their salts are represented in Fig. 1.

We need consider only the one broad powerful line near 1700 cm.⁻¹. This is present in acetic and propionic acids (and in all unionized carboxylic acids yet studied). This frequency vanishes on ionization of the carboxyl group (as is shown for acetic and propionic acids in Fig. 1).

Raman Frequencies of Fatty Acids and Amino Acids.

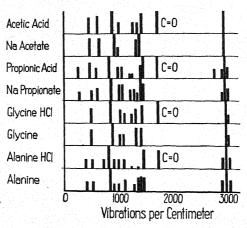


Fig. 1. The Raman frequencies are plotted in reciprocal wave lengths (cm.⁻¹). The height of each line is roughly proportional to its relative intensity. The data are from (Edsall, J. T., J. Chem. Phys., 4, 1 (1936)).

Similarly, on passing from an amino acid hydrochloride to the free amino acid, the strong frequency near 1700 cm.⁻¹ also disappears. (The data for glycine and alanine are typical for several other amino acids that have been investigated.) It follows that the carboxyl group in the isoelectric amino acid is ionized, the molecule being a dipolar ion.

The same conclusion is reached from the comparison of the amino acids and their sodium salts with amines and their hydrochlorides (10). Free amines give one or two strong Raman lines in the range 3300 to 3400 cm.⁻¹, which vanish when the amine is converted into its hydrochloride. The sodium salts of the amino acids show a strong line in this range, of which there is no trace in the free amino acid. Here again the spectroscopic evidence points unmistakably to the dipolar ionic structure.

(2) Ultra-Violet and Infra-Red Spectra. The ultra-violet absorption spectra (11) of amino acids and their salts, when compared with those of fatty acids, amines, and their salts, likewise furnish strong evidence for the dipolar ion structure of the amino acids. Similar evidence from infra-red absorption bands (12) has recently been forthcoming with respect to taurine and glycine.

3. Dielectric Constants and Dipole Moments

The concept of the dielectric constant is fundamental to all electrical theory. Consider two electrically charged bodies (small enough to be treated as points), carrying charges of magnitude q_1 and q_2 , and separated by a distance r. Then the force between them is found to be

$$F = \frac{q_1 q_2}{Dr^2}$$
 (Coulomb's law) (13)

where D, the dielectric constant, is a factor depending on the nature of the surrounding medium. If the dielectric constant is defined as unity for a vacuum, it is found to be greater than unity for all other media.

Another method of measuring D, which is the basis of most of the experimental determinations of this quantity, involves the measurement of the capacity of a condenser. A condenser in its simplest form consists of two conducting plates, separated by a non-conducting medium. If one plate carries a charge +q, the other a charge -q, then a difference of potential, V, will be found to exist between them. The ratio q/V is characteristic of the condenser, and is known as its capacity.

Cavendish—and later, independently, Faraday—discovered that the capacity of a condenser is markedly dependent on the nature of the medium between the plates. If the plates are in water, the capacity is about eighty times as great as in a vacuum; and this ratio is a measure of the dielectric constant of water. If C_0 is the capacity of a condenser *in vacuo*, and C its capacity in some other medium, the ratio C/C_0 is the dielectric constant of the medium.⁴

The dielectric constants of some typical substances are listed in Table II. We may note the very low values for the hydrocarbons,

³ The electrostatic unit of charge is one which, placed one cm. from an equal charge in vacuo, attracts or repels it with a force of one dyne.

It can readily be shown from electrical theory that the two definitions of the dielectric constant given here are equivalent. Either can be derived from the other.

the relatively high values for the alcohols, the very high values for water, hydrocyanic acid, and especially for aqueous solutions of glycine (and other amino acids). What correlations can be established between the chemical structure of these substances and their dielectric constants?

(1) Induced Dipoles. The fundamental clue to the answer was given by Faraday, who assumed that a dielectric medium increases the capacity of a condenser because the molecules of the medium become polarized in the electric field between the plates. This

TABLE II

Dielectric Constants and Dipole Moments of Certain Important Substances

Substance	Dielectric Constant D at 20°	Dipole Moment $\mu \times 10^{18}$ e.s.u.	
Vacuum	1.00		
Hexane	1.87	0	
Octane	1.96	0	
Benzene		04	
Toluene	2.39	0	
Diethyl ether	4.33	1.15	
Chloroform		1.15	
Acetone	21.4	2.75	
Ethanol	24	1.7	
Methanol		1.7	
Water		1.9	
Hydrocyanic Acid	116	2.6	
2.5 M Glycine in water		(15)	

(See Hildebrand, J. H., Solubility 2nd ed., New York, 1936, Chapter IV.) The value for glycine solution is estimated from data concerning this substance the references for which may be found in Table 4.

polarization may be thought of in terms of the effect of the field on the molecules, whereby they become electric doublets, or dipoles (Fig. 2). A dipole is a structure which is electrically neutral as a whole, but in which the "center of gravity" of positive charge is at one point, the center of negative charge at another. If the magnitude of the positive charge is +q, that of the negative charge -q, and d is the distance between them, then the product, qd, is known as the moment (μ) of the dipole.

In terms of modern ideas of atomic structure, it is easy to see how this comes about. Every molecule is made up of atoms with heavy positively charged nuclei, surrounded by light negatively charged electrons. When the molecule is placed between the charged plates of a condenser, the electrons will be pulled toward the positive plate, the nuclei toward the negative plate. The molecule is thus somewhat distorted from its normal condition, and becomes a dipole. The effect of this is to weaken the field; provided of course that the charge on the plates remains constant. This is due

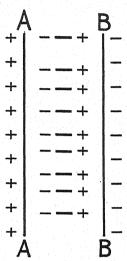


Fig. 2. Induction of dipoles in an electric field between the plates AA and BB of a condenser.

to the fact that the dipoles will themselves give rise to an electric field in a direction opposing the field set up by the plates of the condenser; as may be seen from Fig. 2. Through this weakening of the electric field, the potential difference between the plates (that is, the work done in transporting a unit charge from one plate to the other) is diminished. This is another way of saying that the capacity of the condenser is increased (since capacity = (charge on plates)/(potential between plates), and the denominator of this expression has been reduced, while the numerator remains constant).

Up to this point, it has been assumed that the molecules are polarized only by distortion in the electric field. Such an assumption is sufficient to explain the behavior of many molecules, of which methane (CH₄) may be taken as a simple example. Here the positive charges are represented by the carbon nucleus and the four hydrogen nuclei. On account of the tetrahedral symmetry in the positions of the latter, their center of gravity coincides with the carbon nucleus. The negative charges (electrons) in the molecule

are also, on the average, distributed symmetrically about the central carbon nucleus. The centers of gravity of the positive and negative charges thus coincide, and the molecule is non-polar; that is, it is not in itself a dipole, but only becomes so in the presence of an external field of force. All the aliphatic hydrocarbons, as well as benzene, carbon dioxide, and certain other molecules of high symmetry, are non-polar.

(2) Permanent Dipoles. Many molecules, however, are electric dipoles even in the absence of an external field. Water is a typical example. X-ray and electron diffraction studies have shown that the two hydrogen atoms do not lie in a straight line with the oxygen, but that the molecule is bent, the angle between the two O-H bonds being approximately 105° (13). The two hydrogen nuclei carry each a charge $+e^{5}$ which tends to attract electrons. However, the oxygen nucleus carries a charge equal to +8e, and thus exerts a much more powerful pull on the electrons in the molecule than do the hydrogen nuclei. The region around the oxygen atom thus becomes a center of negative charge, while the two hydrogen atoms are left with a net positive charge, the center of gravity of positive charge lying midway between them.

A permanent dipole such as water will undergo some distortion in an electric field, as would a non-polar molecule like methane. Much more important, however, is the tendency of polar molecules to orient themselves in an electric field. Thus a water molecule, between the plates of a condenser, will tend to set itself with the positive end (the hydrogens) pointing toward the negative plate, the negative end (the oxygen) pointing toward the positive plate.

More generally, these relations may be formulated as follows: in the presence of an electric field of intensity F, an electric moment $\alpha_0 F$ will be induced in a molecule by distortion. The constant of proportionality, α_0 , depends on the ease with which the electrons in the molecule can be displaced, and is known as the molecular polarizability. If the molecule also possesses a permanent electric moment, μ , it will tend to set itself with this moment parallel to the field, but this tendency will be opposed by the thermal agitation of the molecules. Debye (14) has shown for gases in which the molecules are far apart that the average moment per molecule in the direction of the field F, due to orientation is $\mu^2 F/3KT$; 6 that is,

⁵ Where -e is the charge on an electron = 4.77×10^{-10} electrostatic units or e.s.u.

⁶ K = R/N is Boltzmann's constant = 1.371 \times 10⁻¹⁶ ergs per degree.

it is proportional to the square of the permanent moment and to the reciprocal of the absolute temperature. The total moment in the direction of the field, m, is thus

$$m = \left(\alpha_0 + \frac{\mu^2}{3KT}\right)F \tag{14}$$

per molecule, and the moment per mole of gas is N times as great. If the field is of unit strength (1 e.s.u. = 300 volts/cm.), then the total induced moment per mole in the direction of the field, multiplied by the factor $4\pi/3$, is known as the molar polarization, P. For gases, the polarization is very simply related to the dielectric constant, D, by the formula

$$P = \frac{4\pi}{3} N \left(\alpha_0 + \frac{\mu^2}{3KT} \right) = \frac{D-1}{D+2} V \tag{15}$$

when V is the molal volume of the gas. This equation, for substances in which $\mu=0$, was first derived by Clausius and Mosotti. The form given here, which applies also to polar gases, was given by Debye (14). It will be seen from equation (15) that the dielectric constant or the polarization of a non-polar gas, like H_2 or N_2 , at constant volume, is independent of temperature. In substances such as dipolar ions, on the other hand, the moment induced by distortion, $\alpha_0 F$, is very small compared to the permanent moment. In such cases, the polarization, at constant temperature, is directly proportional to the square of the permanent moment.

If a small number of polar molecules be dissolved in a large amount of a non-polar solvent, the polar molecules are generally almost as independent of one another as if they were in a gas. For such systems, Debye (14) has developed an extended form of equation (15), which permits the calculation of the electric moment of a polar solute from measurements of the dielectric constant of its solution in a non-polar solvent (14, 15, 16).

The order of magnitude of the electric moments we should expect to find in simple molecules may be readily estimated. The charge on the electron (-e) is 4.77×10^{-10} e.s.u., and the distances between atoms in a molecule are of the order of magnitude of one Ångström unit $(=1\times10^{-8}$ cm.). A dipole containing charges +e and -e, separated by 1Å, should, therefore, have a moment $(4.77\times10^{-10})\times10^{-8}=4.77\times10^{-18}$ electrostatic units. Experimentally determined values for a number of molecules are given in Tables II and III. It will be observed that the values range from 0 (for the

hydrocarbons) up to 4.8×10^{-18} e.s.u. (for symmetrical dimethylurea).

(3) Dipolar Ions. It is impossible to determine the electric moments of substances like the amino acids by the methods men-

Table III

Dielectric Increments and Dipole Moments of Certain Molecules
(All values are at 25°)

Substance	$\delta = dD/dC$ in water	Dipole moment, p
Methanol(*)	- 1.4	1.65
Ethanol(*)	-2.6	1.7
<i>n</i> -Propanol(*)	- 4.0	1.7
iso-Propanol(*)	- 4.3	1.7
Butanol (tertiary)(*)	- 6.3	1.7
Acetone	-3.2	2.75
Ether(†)	- 7.1	1.1
Dioxane(‡)	- 8.3	0.0
Glycol(*)	- 1.8	2.3
Glycerol(*)	- 2.6	?
Mannitol(*)	- 2.6	?
Sucrose(*)	- 7.5	?
Aniline(20)	- 7.6	1.52
Phenol(20)	- 6.6	1.65
Methyl acetate (20)	- 5	1.75
Pyridine(20)	- 4.2	2.2
Nitromethane(20)	- 2.0	3.05
Acetonitrile(20)	- 1.7	3.3
Acetamide(23)	- 0.8	3.8
Dimethylurea (Symmetrical)(20)	+ 3.0	4.8
Urea(21)	+ 2.7	?
Malonamide(23)	+4.3	?
Glycine anhydride(23)	-10	?
Glycine(22)	+22.6	about 15
α -Amino- n -valeric acid (22)	+22.6	about 15
Diglycine(22)	+71	about 25

Values of μ taken from table in *Trans. Faraday Soc.*, 30, 1934, except those for amino acids, which are estimated by methods discussed in text.

tioned above. They decompose on melting and are insoluble in all non-polar solvents. Information of the utmost importance has been obtained, however, by dissolving them in polar solvents (such as water or one of the alcohols) and studying the dielectric constant of the solution (24). The description of the results is facilitated

^{*} Calculated δ-values are based on the dielectric constant data of Akerlöf (17).

[†] Based on data given by Falkenhagen (18, p. 149).

[‡] Data of Wyman, given by Scatchard and Benedict (19).

by the fact that the dielectric constant, D, in all dilute solutions, is a linear function of the concentration, C, of amino acid in moles per liter (25). $D = D_0 + \delta c$, where D_0 is the dielectric constant of the pure solvent. For most amino acids, indeed, this linear relation holds up to the highest concentrations investigated. The constant $\delta = dD/dC$ is characteristic of the substance, and related to its polarity. Values for δ for many substances are listed in Tables III and IV.

Table III shows that almost all molecules (excluding dipolar ions), even highly polar ones such as acetone, acetonitrile, and acetamide, give negative δ -values in water. In a homologous series, such as the alcohols, all of which have the same dipole moment, the value of δ decreases progressively as the number of CH₂ groups in the molecule increases. This may be attributed to a "dilution" of the effect of the dipoles by the non-polar hydrocarbon groups. The number of dipoles in a mole of liquid remains constant, but the number per unit volume (which is more significant in determining the dielectric constant) decreases (37).

For molecules of somewhat similar size, Table III shows that the value of δ tends to increase with the dipole moment. Of all the molecules listed (excepting dipolar ions), only malonamide, urea, and symmetrical dimethylurea show positive δ -values. The moment of the first two is not definitely known, but that of symmetrical dimethylurea is 4.8×10^{-18} , δ being +3. On the other hand, glycine (and all α -amino acids) show an extremely high δ -value, about 23. Although the electric moment cannot be exactly calculated from this value, it must, in any case, be much greater than that of dimethylurea. Its order of magnitude may be estimated by considering a simple molecular model. From X-ray diffraction studies (38, 39), the arrangement of the atoms in glycine is known to be approximately as follows (See discussion in (37).

The angle between the C-C and the C-N bonds may be taken as the tetrahedral angle (109° 28′); the angle between the C-O bonds is approximately 125° (40); the C-N distance is very nearly 1.45 Å; the C-C distance, 1.54 Å; C-O in the carboxyl group is 1.29 Å. If we assume that the center of the positive charge in the dipolar ion resides on the nitrogen atom, and the center of the negative charge midway between the two oxygen atoms of the ionized carboxyl group (since the group is presumably symmetrical with respect to the oxygen atoms), then the distance between the two charges is 3.0 Å, and the moment of the dipolar ion is 14.4×10⁻¹⁸e.s.u.

Table IV

Dielectric Increments of Amino Acids and Other Dipolar Ions in Water
(Unless otherwise indicated, the δ-values are at 25°)

Substance	Dielectric Increment, δ	References	
(a) Amino Acids			
Glycine	22.6; 23.0; 26.4; 30	22, 25, 26, 27	
α -Alanine	23.2; 23.6; 27.7	22, 25, 26	
α -Amino- n -butyric acid	23.2	$22^{'}$	
α -Amino- n -valeric acid	22.6	22	
<i>d,l</i> -Valine	25	23	
l-Leucine	25	23	
d, l -Proline	21	23	
Sarcosine	24.5	23	
d-Glutamic acid	26	23	
l-Aspartic acid	27.8	26	
$d ext{-Glutamine}\dots\dots\dots\dots\dots$	20.8	28	
l-Asparagine	28.4; 20.4	26, 28	
N-Phenylglycine	ca. 30	29	
Glycocyamine	30	28	
Creatine	32.2	28	
β-Alanine	34.6; 35; 42.3	22, 30, 25	
β-Aminobutyric acid	32.4; 36	22, 31	
Taurine	41	23	
γ-Aminobutyric acid	51	30	
γ -Aminovaleric acid	54.8	22	
Ornithine	51	31	
δ-Aminovaleric acid	63	30	
Arginine		32	
Acetylhistidine	62	28	
e-Aminocaproic acid	77.5; 73	22, 30	
ζ-Aminoheptylic acid	87	30	
(b) Peptides			
Diglycine	70.6; 70; 70.5; 80	22, 23, 33, 27	
Glycylalanine		28	
Alanylglycine	71	28	
Leucylglycine	62, 68.4	33, 28	
Glycylleucine	74.6; 70	28, 33	
N-Methyl leucylglycine		28	
Glycylphenylalanine		28	
Phenylalanylglycine	. 56.7	28	

TABLE IV-Continued.

Substance	Dielectric Increment, δ	References	
(b) Peptides (continued)			
Triglycine	113; 128	22, 33	
Leucylglycylglycine	120.4; 112	28, 33	
Tetraglycine	159	22	
Pentaglycine	215	22	
Hexaglycine	234	22	
Heptaglycine	290 (in 5.14 molar urea)	34	
(c) Double Dipoles			
e, e'-Diguanido-di(α-thio-n-caproic acid).	151	28	
e, ε'-Diamino-di (α-thio-n-caproic acid)	131	35	
Lysylglutamic acid	345	35	
(d) Aliphatic Betaines			
Betaine (N-trimethyl glycine)	24-27; 18.2	31, 36	
Betaine of δ -amino- n -valeric acid	60	27	
Betaine of z-amino-pentadecyclic acid	220 (at 70°)	27	
Betaine of π -amino-heptadecyclic acid	190 (at 80°)	27	
(e) Aromatic Betaines			
Pyridine betaine	18.5; 20.5	36, 27	
Ortho benzbetaine	18.7; 20	36, 29	
Meta benzbetaine	48.4; 58	36, 29	
Para benzbetaine	72.4; 68; 62	36, 29, 27	

The order in which the δ -values for any substance are listed corresponds to the order of the references from which the values were obtained.

Lindquist, F. E., and Schmidt, C. L. A. Compt. rend. Lab. Carlsberg, 22, 307 (1938) have obtained the following values for δ : Glycine (25°) 22.93, (0°) 26.28; Alanine (25°) 23.49, (0°) 26.67; Hydroxyproline (25°) 20.48, (0°) 23.31; Proline (30°) 21.47, (1.9°) 23.36.

The temperature coefficients for the δ-values are:

Glycine: $\delta = 26.28 - 0.134 t$

Alanine: $\delta = 26.67 - 0.127 t$

Hydroxyproline: $\delta = 23.31 - 0.163 t + 0.002 t^2$

See also, Wyman (34) and McMeekin and Wyman (22). For dielectric constants of the proteins of horse serum see Ferry, J. D., and Oncley, J. L., J. Amer. Chem. Soc., 60, 1123 (1938); and for carboxyhemoglobin see Oncley, J. L., J. Amer. Chem. Soc., 60, 1115 (1938).

Estimates of the same order of magnitude have been made by Wyman (34) from a theoretical analysis of the dielectric constant data for amino acids, and by Kirkwood (41) from a theoretical treatment of the solubility of glycine in salt solutions. It seems fairly certain then that, while this estimate may be in error by 15 or 20 per cent, it cannot be very far from the true value. Thus the

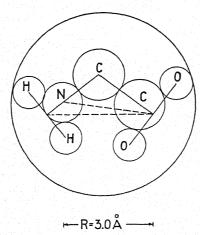


Fig. 3. Steric model of glycine. (Cohn, E. J., Annual Rev. Biochem., 4, 97 (1935).)

moment of glycine is approximately eightfold that of water, one of the most polar solvents known.

In the amino acids and peptides with a greater separation between the amino and carboxyl groups, the values of δ are much greater (Table IV). Glycylglycine has a δ -value of 70, ϵ -aminocaproic acid, 77; glycine heptapeptide, 290; and lysylglutamic acid, with two free amino and two carboxyl groups, 345. In concentrated solutions of such substances, then, the dielectric constant is far greater than in any solution not containing dipolar ions. The effect of such highly polar media on the activities and rates of reaction of all charged bodies within them must be profound. It is still difficult, however, to state exactly what is the relation between the observed value of δ and the electric moment of the molecule. Even the simplest molecule of this type, β -alanine, (+H₃N·CH₂·CH₂·COO⁻), can exist in many different shapes. Two extreme forms are shown in Fig. 4.

In Fig. 4a, the distance between the positive and negative charges is maximal (about 4.4 Å); in 4b, it is minimal (about 2.9 Å).

An infinite number of intermediate forms is possible through rotation of the C-N and CO bonds about the axis of the adjoining C-C linkage. The average state of the molecule is probably represented by one of these intermediate positions.⁷

As the number of intervening atoms between the amino and carboxyl groups increases, the number of possible molecular configurations increases very rapidly. At one extreme (maximal moment) the intervening atoms will be stretched out in an ex-

Fig. 4. Two configurations of the β -alanine molecule. (The hydrogen atoms of the CH₂ groups are omitted.)

tended zigzag chain, as in the crystals of the fatty acids and paraffins (42); at the other extreme, they will bend around to form a ring, the carboxyl and amino groups touching as closely as their own dimensions will permit. Here again the most probable configuration of the molecule will be an intermediate one.⁸

One thing at least, however, is clear from the dielectric data. The value of δ increases steadily with increase in the number of atoms between the NH₃+ and the COO⁻ group. Certainly, therefore, the electric moment must increase also. Hence, the average state of the molecules must be very far from the ring structure.⁹

The possibility that the molecules exist in an extended rod-like form cannot be excluded with equal assurance. It was at first supposed indeed that this assumption afforded the best explanation of the observed δ -values (22), but further analysis of the problem (34, 45, 46) has led to the belief that the δ -value may be taken as roughly proportional to the molar polarization, P, of the solute and hence to the square of its dipole moment (Equation (15)). This assumption would lead to an electric moment for

 $^{^{7}}$ These relations may be readily visualized with the aid of simple molecular models, now readily available.

⁸ For a detailed analysis of this problem, see Kuhn (43) and Guth and Mark (44).

⁹ This, of course, does not exclude the possibility that a small number of molecules may be in the ring form at any one time. Such a configuration must be assumed possible in order to explain such reactions as internal anhydride formation.

heptaglycine of approximately 52 and for lysylglutamic acid, 57.5×10^{-18} e.s.u.

(4) Dielectric Constants and Electric Moments of Proteins. A relatively small protein molecule, such as egg albumin (radius 21 Å), is large enough to possess dipole moments greater than those of any of the peptides yet studied, even if there were only one positively charged group on one side of the molecule and one negatively charged on the other. Actually egg albumin possesses approximately 27 positive and 27 negative groups. If all the positive charges were crowded at one side of the molecule and all the negative charges at the other, the resulting moment would be about 5400×10^{-18} e.s.u.; on the other hand, the component dipoles might be so arranged that their vector sum is very nearly zero, with a very low resultant moment.

The study of the dielectric constants of protein solutions presents formidable technical difficulties. In recent years, however, these have been largely overcome, and it appears probable that reliable and reproducible measurements on protein solutions will soon be obtainable with relative ease. Wyman (47) showed that zein increases the dielectric constant of n-propyl alcohol-water mixtures, and calculated a fairly high electric moment for the molecule. Recalculation of this data leads to a δ-value of approximately 11,000 for zein. Errera (48) obtained δ-values of approximately 30,000 per mole for hemoglobin, and 10,000 for egg albumin. By another method, Shutt (49) obtained for egg albumin the lower value 4,000. Dr. J. L. Oncley has developed in this laboratory a modified bridge method which is well adapted for studying the dielectric constants of protein solutions. He has obtained the value 35,000 (\pm 5000) for hemoglobin and 3400 (\pm 500) for egg albumin, in reasonable agreement with the results of Errera and of Shutt, respectively. These values suggest electric moments which are very large, but probably far smaller than the maximal moments conceivable for molecules carrying so many charges.

(5) Phospholipids as Dipolar Ions. It may be mentioned here that dielectric constant measurements indicate dipolar ionic structure for lecithin and sphingomyelin (27). This might well be expected on chemical grounds since from data on dissociation constants (50, 51) the nitrogen of the choline radical in these molecules should carry a positive charge and the phosphoric acid residue a negative charge. It is significant that proteins and phospholipids, the two most essential elements of chemical structure in the living cell, should possess this type of electrical configuration.

4. Apparent Molal Volumes and the Electrostriction of the Solvent

If Δn moles of a substance be added to a given solvent, the volume of the solvent will change by ΔV cc. The ratio $\Delta V/\Delta n$ at constant temperature and pressure, is known as Φ , the apparent molal volume of the solute; it is the change in volume of the system per mole of substance added. It is estimated by adding a known weight of solute to a known weight of solvent, and measuring the density of the pure solvent and of the resulting solution. Its exact value depends on the concentration of solute in the resulting solution, but for non-electrolytes, it does not vary greatly with concentration and is of the same order as the molal volume of the pure solute.

It has long been known (56) that component atoms and groups make characteristic contributions to the apparent molal volumes of molecules. Thus, pairs of organic isomers are found in general to have nearly identical apparent molal volumes. The latter may, therefore, be approximately calculated as the sums of volumes assigned to component groups.

Dipolar ions show striking and systematic deviations from this general rule. Compare, for example, the values of Φ in water for the isomers, glycollic amide and glycine; lactamide, and α -and β -alanine; and methylhydantoic acid and glycylglycine (Table V).

Table V

Apparent Molal Volumes in cc./mole of Dipolar Ions and Their Uncharged Isomers

(E denotes the difference in each case between charged and uncharged isomers)

	Ф	E	Reference
CH ₂ OH · CONH ₂	56.2	12.7	(*)
+H ₂ N · CH ₂ · COO ⁻	43.5		(57)
$\begin{array}{c} \text{CH}_3 \cdot \text{CHOH} \cdot \text{CONH}_2 \\ + \text{H}_2 \text{N} \cdot \text{CH}(\text{CH}_3) \cdot \text{COO}^- \\ + \text{H}_2 \text{N} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COO}^- \end{array}$	73.8 60.6 58.9	13.2 14.9	(*) (57) (57)
$H_2N \cdot CONH \cdot CH(CH_3) \cdot COOH$	94.2	17.0	(58)
+ $H_3N \cdot CH_2 \cdot CONH \cdot CH_2 \cdot COO$	77.2		(57)

All values of Φ are for concentrations between 0.2 and 0.3 molal. * Denotes unpublished data of T. L. McMeekin.

 10 A closed related quantity, the partial molal volume is the differential increment $\partial V/\partial n$ at constant temperature and pressure. In very dilute solutions, the two quantities are nearly identical. For a thorough discussion of apparent and partial molal volumes, see Lewis and Randall, (52, Chapter IV) and Gucker, (53). Accurate values of apparent and partial molal volumes for many amino acids are given by Dalton and Schmidt, (54 and 55).

The value of Φ is strikingly lower for the dipolar ions than for the isomeric uncharged molecules. Moreover, this lowering (E, in Table V) is greater for β - than for α -alanine, and notably greater for glycylglycine than for glycine. The same statement holds for the order of increasing dipole moments in this group of substances. The electric charging of the molecule is accompanied by a decrease in volume of the system.

This is a general phenomenon; it occurs whenever ions are formed from uncharged molecules. Thus the following figures (59) have been found for the volume changes accompanying the ionization of weak acids and bases:

CH₃·COOH
$$\rightarrow$$
CH₃·COO⁻+H⁺ $\Delta V = -10.3$ cc./mole
CH₃·NH₃OH \rightarrow CH₃·NH₃⁺+OH⁻ $\Delta V = -27.0$ cc./mole
H₂O \rightarrow H⁺+OH⁻ $\Delta V = -21.0$ cc./mole

It will be noted that the volume contractions accompanying ionization are of the same order of magnitude as those involved in the rearrangement of an uncharged molecule to an isomeric dipolar ion. A similar contraction of the system occurs when strong electrolytes are dissolved in water, as is shown by the extraordinarily low, often negative, apparent molal volumes of strong electrolytes.

This volume change must be attributed primarily to the electrical action of the ionic groups in attracting and compressing solvent molecules (60). The effect, known as electrostriction, is large, both because the electric field in the neighborhood of an ion is very intense, and because it is inhomogeneous; it falls off rapidly with increasing distance from the ion. At a distance r from a charge e, the intensity of the electric field in a vacuum is by Coulomb's law e/r^2 . At a distance of 3×10^{-8} cm. from a sodium ion the field strength is thus 160,000,000 volts per centimeter in a vacuum.¹¹ At 10 Ångström units, it is 14,000,000 volts per centimeter (14, p. 111). The dipoles of the solvent will be oriented in this intense field. Thus the negative oxygen of a water dipole will point toward a sodium ion, the positive hydrogens away from it. Thereby the dipoles tend to oppose and weaken the electric field produced by the ion, and to diminish the electrical energy of the system. This diminution will be most marked where the original field is most

¹¹ Charge on electrons = 4.77×10^{-10} electrostatic units. 1 electrostatic unit of intensity = 300 volts per cm. $\therefore (300 \times 4.77 \times 10^{-10})/(3 \times 10^{-8})^2 = 160,000,000$.

intense—that is, in the immediate neighborhood of the ion. Hence the dipoles of the solvent will be concentrated around the ion, and packed much more closely together than in an ion-free solvent.

Table VI

Apparent Molal Volumes (Φ) and Electrostrictions (E) of Amino Acids, Peptides and Related

Compounds in Water

Substance	Φ (cc./mole)	E (cc./mole)	Substance	Φ (ccmole)	E (cc./mole)
(a) Amino Ac	ids		(b) Peptides		
Glycine (57)	43.5	13.5	Diglycine (57)	77.2	16.1
α -Alanine (57)	60.6	12.7	Glycylleucine (28)	139.8	18.7
Serine (57)	60.8	12.9	Leucylglycine (28)	143.2	15.3
α -Amino- n -butyric acid (57)	76.5	13.1	Glycylalanine (28)	93.9	15.7
α -Aminoisobutyric acid (57)	78.1	11.5	Alanylglycine (28)	94.5	14.1
α -Amino- n -Valeric acid (57)	92.7	13.2	Glycylphenylalanine (28)	155.4	15.9
α -Aminoisovaleric acid (57)	91.3	14.6	Phenylalanylglycine (28)	160	11.3
α-Amino-n-Caproic acid (57)	108.4	13.8	Triglycine (57)	113.5	16.1
α -Aminoisocaproic acid (57)	107.5	14.7	Leucylglycylglycine (28)	178.5	16.3
l-Asparagine (28)	78.0	15.3			1
β-Alanine (57)	58.9	14.4	(c) Double Dipoles		
β-Aminobutyric acid (57)	76.4	13.2			
γ -Aminovaleric acid (57)	90.0	15.9	e, e'-diamino-di-(α-thio-n-cap-		
e-Aminocaproic acid (57)	104.9	17.3	roie acid) (35)	226.8	27.1
Lysine (57)	108.5	18.3	Lysylglutamic acid (35)	172.7	38.8
Proline (57)	1	13.8	(d) Aromatic Amino Acids a	and Betai	nes
Hydroxyproline (57)		12.7		i -	T
Phenylalanine (57)		13.7	o-Aminobenzoic acid (57)	96.7	5.9
Histidine (57)		10.8	m-Aminobenzoic acid (57)	90.3	12.3
Acetylhistidine (28)	1	11	p-Aminobenzoic acid (57)	97.3	5.3
Tryptophane (57)	144.1	15.1	Betaine* (36)	97.7	8.2
			o-Benzbetaine* (36)	146.5	4.9
	14.74		m-Benzbetaine* (36)	145.0	6.4
		1.000	p-Benzbetaine* (36)	141.3	10.1
			N-dimethylphenylglycine* (36)	146.8	4.6

* The electrostriction produced by betaines is much greater in ethanol and ethanol-water and ethanol-benzene mixtures than in water (36).

Apparent molal volumes for uncharged organic molecules were calculated by the method of Traube (56) or by a modification of that method described by Cohn, McMeekin, Edsall, and Blanchard (57). The difference between these calculated values and the observed values of Φ give the estimated electrostriction per mole. The values given in this table differ slighly from those given in Table V on account of the slight difference in the methods of calculation employed. The difference is of the order of magnitude of 0.6 cc. per mole which may be taken as the probable uncertainty in the estimated values.

Some very careful measurements of apparent and partial molal volumes in amino acid solutions have been reported by Dalton and Schmidt (54, 55) and by Daniel and Cohn (63). These would lead to slightly revised electrostriction values (by 0.2–0.5 cc./mole) for some substances listed here. Since no equally careful studies on uncharged isomers of amino acids have been made, such a revision is not attempted at this time

Equilibrium is attained when the work done by the electric field in orienting and compressing the solvent exactly balances the reduction in the energy of the field produced by the oriented solvent dipoles.¹² The fact that free ions and dipolar ions produce

¹² For detailed attempts to calculate this work and the resulting compression, see Webb (61) and Zwicky (62).

compressions of the same order of magnitude suggests similar electric field intensities in the neighborhood of both.

In Table VI, values of Φ , and of the electrostriction, E, are tabulated for a number of amino acids and peptides. For most of these substances, no uncharged isomers are available for comparison. Values of Φ for such uncharged isomers may be calculated from the known increments in Φ due to the groups in the molecule. Thus it is known that in homologous series of compounds each additional $-CH_2$ group increases the value of Φ by approximately 16.3 cc./mole; likewise, similar compounds differing by one -CONH- group differ in Φ -values by 20.0 cc./mole (57). Making use of other such increments as determined by Traube (56), the value of Φ (uncharged) may be calculated for any of the substances listed in Table VI. The difference between the Φ -value so calculated and Φ (observed) yields the electrostriction, E.

The electrostriction produced by the α -amino acids ranges between 11.5 and 14.7 cc. (Average about 13.3 cc.) The betaines, with three organic groups on the charged nitrogen atom, give much lower values; presumably because the bulky methyl or phenyl groups prevent close packing of the solvent molecules in the immediate neighborhood of the charge. The β -, γ -, and ϵ -amino acids give higher E-values than any of the α -acids, because of the greater separation of the charged groups. The same is true of all the peptides. As the charged groups become more and more widely separated, the electrostriction appears to approach asymptotically a certain limiting value, in the neighborhood of 20 cc./mole for compounds containing one -NH₃+ and one -COO- group. In lysylglutamic acid, with two positive and two negative groups. widely separated, the electrostriction is nearly twice as great (38.8 cc.). The values for the aminobenzoic acids show that the meta acid exists chiefly as a dipolar ion, the ortho and para acids chiefly as uncharged molecules.

Proteins in water should also produce electrostriction, but the effect will be difficult to detect. Thus a molecule like egg albumin, with approximately 27 positive and 27 negative groups, should produce electrostriction amounting to about $27 \times 20 = 540$ cc./mole. The apparent molal volume of egg albumin is about 25,000 cc./mole; the contraction due to electrostriction thus calculated would be only about 2 per cent.

The electrostriction effect may prove significant in certain biological processes. Thus the studies of Schmengler and of Höber

(64) indicate that dipolar ions pass much less readily through artificial membranes with very fine pores and through the kidney tubules than do isomeric uncharged molecules. These authors suggest that the shell of water dipoles clustered around the charged groups increases the effective volume of a dipolar ion and prevents it from passing through an opening in a membrane which would allow the passage of an uncharged isomer. Other effects of the electric charges on the amino acids may also be involved in these phenomena; and the further development of this field should lead to interesting results.

SECTION II. SOLUBILITY OF AMINO ACIDS, PEPTIDES AND RELATED SUBSTANCES

5. Melting Points and Crystal Structure of Amino Acids

(1) Solubility in Water. The amino acids are remarkable among organic compounds for their very high melting points. All of them melt (with decomposition) above 200°—most of them in the neighborhood of 300°. Melting points of most inorganic salts are far higher than this, while those of the great majority of organic compounds are very much lower (see Table VII and also Chapter IX, Section XI). Among uncharged compounds the amides have unusually high melting points; oxamide, the diamide of oxalic acid, with a density of 1.667 melts at 419°.

Ionic compounds are held in a crystal lattice by very powerful electrostatic forces, and their melting points are correspondingly high. Non-polar or slightly polar organic compounds are held by much weaker van der Waals forces and have correspondingly lower melting points. The isoelectric amino acids are intermediate between the two extreme types of crystal lattice just described. Bernal (39) has concluded that "for the simpler amino acids the tendency will be to approach ionic packing (as witnessed by their high specific gravity). . . . The determining factor in the structure of α -amino acids is the dipole group,

These groups pack together in double molecules or in extended chains." On the other hand, the presence of hydrocarbon chains and other organic radicals in the amino acids produces necessarily

TABLE VII

Melting Points of Salts, Amino Acids and Uncharged Molecules

Inorganic Salts	Melting Point	Normal Hydrocarbons	Melting Point
	degrees		degrees
Barium Chloride	925	Ethane	-172
Sodium Chloride	804	Propane	-189
Potassium Chloride	772	Pentane	-131
Lithium Chloride	613	Hexane	- 94
	547	Heptane	- 90
Lithium Bromide	547 446	Octane	- 56
Amino Acids		Aliphatic Amines and Acids	
Glycine	290	Ethylamine	- 80.6
d,l-Alanine	297	Propylamine	-83.0
<i>d,l</i> -Valine	292	Amylamine	-55.0
<i>d,l</i> -Leucine	332	Hexylamine	-19.0
d, l - α -Aminocaproic Acid	327	Acetic Acid	16.6
Diglycine	262-264	Propionic Acid	-22.0
d,l-Phenylalanine	318-320	Valeric Acid	-34.5
<i>l</i> -Tyrosine	342-344	Caproic acid	- 9.5
Certain Amino Acid		Certain other	
Derivatives		Organic Compounds	
Glycyl Amide	66- 68	Acetamide	81
Alanyl Amide	71- 72	Glycolamide	119
Leucyl Amide	105-108	Lactamide	77
Formylglycine	152-153	Butyramide	116
Formylglycylglycine	168-170	Benzamide	130
Formyl-α-aminobutyric		Acetanilide	114
acid	154-155	Acetnaphthalide	159
Formylleucine	114-115	Phenylthiourea	154
Acetylglycine	206	Urethane	48
Hydantoic acid of		Ethyl Allophanate	191
glycine	169-170	Glucose	146
Hydantoic acid of	-00 110	Sucrose	186
alanine	169-170	Urea	133
Hydantoin of glycine	217-218	Formyl Urea	169
Hydantoin of α-amino-	~1. 210	Biuret	193
butyric acid	118-120	Acetyl Urea	217
Hydantoin of leucine	208-209	Carbonyl diurea	233
Hydantoin of aspartic	200-209	Allantoin	$\begin{array}{c} 235 \\ 235 \end{array}$
acid	215-217		194
auu	410-417	Acetyl biuret	194

Most of the data on this table have been given by McMeekin, Cohn, and Weare (58). Other data not appearing in their table have been derived from International Critical Tables, Volume I. The values of glycolamide and lactamide have been determined by Dr. T. L. McMeekin. For further data and discussion on melting points of amino acids, see Chapter IX, Section XI.

a crystal structure more complicated than that of purely ionic crystals (see Chapter IX, Section VIII).

The forces binding molecules or ions in the crystal lattice exert a profound influence on solubility. Other things being equal, molecules bound very firmly in a crystal lattice will be less soluble than molecules otherwise similar but forming a looser type of lattice. Solubility is determined partly by the forces acting between molecules of the pure solute, partly by the affinity between solvent and solute. Thermodynamically these two components may be separated, following a treatment given by Butler and his collaborators (65). Any substance, at a given temperature, may be regarded as exerting a definite vapor pressure p° ; even though, as in the case of the amino acids, this pressure is extremely small. 13 The saturated vapor of the solute must then be in equilibrium with the pure solute and also with its saturated solution in any solvent. If Henry's law holds for the equilibrium between the vapor and the solution, then the amount dissolved is proportional to the vapor pressure, and at saturation:

$$N = Kp^{\circ} \tag{16}$$

N, the solubility (here expressed as mole fraction) is thus the product of two factors. One, the vapor pressure, p° , depends only on the nature of the intermolecular forces in the pure solute. The other, K, determines the distribution coefficient of the solute between the vapor phase and the solution; it is a measure of the affinity between solvent and solute.

In many cases the ratio (which we may call K_0) of the mole fraction of solute in a very dilute solution, to its vapor pressure p above such a solution, is not the same as K for a saturated solution. (In such cases, of course, the activity coefficient of the solute varies with its concentration or mole fraction.) In such cases the product K_0p° gives an "ideal" or corrected solubility value; and in this modified form equation 16 may be applied whether the solute is a liquid or a solid, even when it is miscible with the solvent in all proportions. Table VIII, calculated from the work of Butler and his collaborators, gives values of K (or K_0 , where this differs from K) and p° for a number of organic molecules.

¹³ Amino acids may be sublimed *in vacuo* with little decomposition at temperatures around 150° (66), and must therefore exert a measurable vapor pressure at these temperatures. Knowledge of this vapor pressure and of the heat of sublimation should permit the calculation of the vapor pressures at lower temperatures.

Table VIII

Vapor Pressures, and Distribution Coefficients Between Vapor and Aqueous Solution,
for Certain Organic Molecules

Substance	Vapor Pressure (p°) mm. Hg $t = 25^{\circ}$ unless otherwise indicated	$K = (N/p) \times 10^3$	
GlycolGlycerol		392.0 1410.0	
Acetic AcidPropionic Acid	11.7 (20°) 0.75 (20°)	79.5 54.0 44.5	
Methyl Alcohol Ethyl Alcohol n-Popyl Alcohol n-Butyl Alcohol n-Amyl Alcohol n-Hexyl Alcohol n-Heptyl Alcohol n-Octyl Alcohol	122.2 59.0 20.1 6.78 2.50 0.719 0.224 0.083	5.42 4.59 3.44 2.78 1.83 1.549 1.253 0.981	
Ethylamine	724 (15.5°) —	2.38 1.89 1.565	
Acetone	229.2	0.720	
<i>n</i> -Propionitrile		0.634 0.457	
Methyl AcetateEthyl AcetatePropyl Acetate	170 (20°) 72.8 (20°) 33	0.261 0.179 0.118	
Diethyl Ether Ethyl Propyl Ether	537 178	0.0287 0.0208	
Methane. Ethane. n-Butane.		0.000031 0.000042 0.000020	

All data from Butler (65) except a few of the p° values, which are from International Critical Tables Vol. III.

The data of Table VIII show that high solubility in water may be due either to a high p° or a high K-value. Thus glycerol is miscible with water in all proportions, although the vapor pressure is very low, because of its high K-value; while ethylamine with a

very moderate K-value is also completely soluble in water because of its high vapor pressure. Diethyl ether, which also has a high vapor pressure, has such an extremely low value of K that its solubility in water is relatively small.

In the homologous series listed, each additional CH2 group reduces p° nearly threefold, and K by about one third. The slight affinity of the hydrocarbons for water is reflected in the exceedingly low K-values for methane, ethane, and butane. Glycerol, with its three hydroxyl groups, shows an affinity for water more than ten million times as great. The other substances are intermediate. Although all the polar molecules show far higher K-values than those for the non-polar hydrocarbons, there is certainly no direct relation between the value of K and polarity. Thus K for acetic acid is more than one hundred times as great as that for propionitrile, although its electric moment is less than half as large. The substances possessing free hydroxyl, carboxyl, or amino groups show in general a much higher affinity for water than other molecules of nearly the same dipole moment. Hildebrand (1, 67) has called attention to these relations on the basis of other experimental data and concluded that the distinctive character of these groups is due to their ability to form hydrogen bonds with the solvent water.

Amino acids and proteins are rich in carboxyl, hydroxyl, and amino groups, and hydrogen bonds may well play a significant rôle in their solubility behavior, ¹⁴ but because they are dipolar ions with very great dipole moments, their electrical structure is probably more important. Uncharged isomers of amino acids, such as glycolamide and lactamide, although also rich in hydrogen bonds, melt at a much lower temperature (Table VII) and show markedly different solubility behavior (Table XIV).

Amino acids, as indicated by their high melting points, must have very low vapor pressures. Hence according to equation (16), they will have a very low solubility in all solvents for which the value of K is not unusually high; that is, in all non-polar solvents. The high solubilities of many amino acids in water thus indicate extremely high K-values for this solvent, probably surpassing that given for glycerol in Table VIII.

¹⁴ A theory of protein structure and denaturation, in which the hydrogen bond is given a very important place, has recently been proposed (68).

Change in the solubility, N, with temperature is related to the heat of solution, ΔH , by the equation of van't Hoff:¹⁵

$$\frac{d \ln N}{dT} = \frac{\Delta H}{RT^2} \tag{17}$$

The heat of solution, like the solubility, may be resolved into two factors: the heat of sublimation, ΔH_s , or of vaporization, ΔH_v , and the heat of solvation, ΔH_m , of the vapor in the solvent.

$$\Delta H = \Delta H_s + \Delta H_m;$$
 or $= \Delta H_v + \Delta H_m$ (18)

Here again, one term depends only on the properties of the pure solute; the other on the interaction between solvent and solute.

For a solid, such as an amino acid, the heat of sublimation determines the change of the vapor pressure with temperature:¹⁶

$$\frac{d \ln p^{\circ}}{dT} = \frac{\Delta H_{\bullet}}{RT^{2}} \tag{19}$$

In general, substances such as the amino acids, which melt at very high temperatures will show very low values of p° and high values of ΔH_s as compared with most organic compounds.

Molal heats of vaporization for many substances may be approximately calculated as the sum of the binding cohesive forces due to various groups in the molecule. Meyer and Mark (71) have given a table of such cohesive energies for various groups, which is reproduced here (Table IX).

The high values for the hydroxyl and carboxyl groups are again notable; but the very great cohesive energy of the -CONH₂ and -CONH- groups is outstanding. The cohesive energy of the peptide linkage, together with the charged groups of dipolar ions, must play a major rôle in determining the crystal lattice energy and the solubility of peptides and proteins. Thus Emil Fischer (72) pointed

¹⁵ This equation holds strictly only if the activity coefficient, f, of the solute is independent of concentration. Where this is not the case, we may use the equation of Brönsted (69). See also Dalton and Schmidt (54).

$$\Delta H = RT^2 \left(\frac{d \ln N}{dT} + \frac{d \ln f}{dN} \frac{dN}{dT} \right)$$

¹⁶ Closely related to the heat of sublimation is the crystal lattice energy $\Delta E = \Delta H_s - RT$, which is the energy required to separate the molecules in the crystal lattice completely from one another. The lattice energy of inorganic crystals has been the object of extensive investigations by Born, Haber, Fajans, and others. See review by Sherman (70).

out that the solubility of the glycine peptides in water decreases with increasing number of glycyl residues in the chain. The high symmetry of these peptides and the absence of side chains, permits very close packing of the molecules in the crystal; the high cohesive forces due to the -CONH- groups, combined with the electrostatic forces due to the charged groups, make the crystal lattice energy high and the solubility low.

Heats of sublimation and crystal lattice energies have not as yet been directly determined for amino acids or peptides. However,

Table IX

Molecular Cohesion of Various Aliphatic Groups

Group	Molecular Cohesion in cal. per mole	Group	Molecular Cohesion in cal. per mole	
$ \begin{array}{c} -\operatorname{CH_3} \\ =\operatorname{CH_2} \end{array} $	1780	-NH ₂ -Cl	3530 3400	
$ \begin{array}{c} -\mathrm{CH_2} - \\ =\mathrm{CH} - \end{array} $	990	less exactly known		
-CH-	-380		1	
-0-	1630	$-\mathbf{F}$	2060	
-OH	7250	-Br	4300	
=CO	4270	-J	5040	
-CHO	4700	$-\mathrm{NO}_2$	7200	
-COOH	8970 —SH		4250	
$-COOCH_3$	5600 -CONH ₂		13200	
$-\mathrm{COOC_2H_5}$	6230	-CONH-	10600	

(Meyer, K. H., and Mark, H., Der Aufbau der hochpolymeren organischen Naturstoffe, Leipzig, 1930.)

extensive and accurate studies of solubility in water at various temperatures between 0° and 100° have been reported by Dalton and Schmidt (54, 55) and by Dunn, Ross, and Read (73); and heats of solution, which are so intimately related to the temperature coefficient of solubility (Equation 17), have also been determined in many cases (54, 55, 74). The available data are compactly summarized in Chapter XV, Table IX (Solubility) and Table V (Heats of Solution).

Comparison of the heats of solution of closely related substances may be used to gain some information regarding differences in crystal lattice energy. This is particularly true when optically active and racemic forms of the same compound are considered. The heat of solvation (ΔH_m in equation 18) is presumably the same

 ${f T_{ABLE}~X}$ Heats of Solution 1 and Crystal Densities of Racemic and Optically Active Amino Acids

Substance	Crystal Density	Heat of Solution ΔH : Cals/mole	$\Delta H_{d,l} - \Delta H_{\text{act.}}$	Ref.
d-Alanined,l-Alanine	1.401 1.425	1830 2200	370	(54) (54)
<i>d</i> -Valine	1.230 1.316	500 1590	1090	(75) (54)
l-Leucined,l-Leucine	1.165 1.191	830 2070	1240	(54) (54)
d-Isoleucine d , l -Isoleucine		843 1780	937	(55) (54)
d-Phenylalanine d , l -Phenylalanine		2820 2760	-60	(55) (54)
l-Tyrosine d-Tyrosine d,l-Tyrosine	1.456	5950 5950 6110	160	(76) (76) (76)
d,l-Diiodotyrosine l-Diiodotyrosine	-	5830 7830	-2000	(76) (76)
l-Aspartic acidd,l-Aspartic acid	_	5580 6500	920	(54) (54)
d-Glutamic acid d , l -Glutamic acid	1.538 1.460	6050 5710	-340	(54) (54)
α -Amino- n -butyric acid α -Amino-iso-butyric acid	1.231 1.278			

The references given in the table are to the data on heats of solution. The density data are all taken from (77), except those for *l*-tyrosine and *d*- and *d*, *l*-glutamic acid, which are from International Critical Tables, Volume I. The heats of solution tabulated are the "corrected" values given by Dalton and Schmidt (54). The work of Zittle and Schmidt (74) has indicated that certain of these values should probably be revised, but the values given here are apparently the most directly comparable for the racemic and optically active forms of the different substances.

¹ See also Chapter XV, Table V.

for the d- and l-forms of any substance (provided the solvent is not optically active). Therefore, in dilute solution, it should also be the same for the d,l-form, since this in the vapor state will consist simply of equal numbers of d- and l-molecules. Hence the difference in heat of solution between the two forms should reveal directly

the difference in their heats of sublimation or crystal lattice energies:

$$(\Delta H)_{d,l} - (\Delta H)_{\text{act.}} = (\Delta H_s)_{d,l} - (\Delta H_s)_{\text{act.}} = (\Delta E)_{d,l} - (\Delta E)_{\text{act.}}$$
(20)

The available data, summarized in Table X, reveal that there is generally a marked difference in heat of solution between the d- or l- and the d,l-form of an amino acid. In six of nine substances listed, the d,l-form shows a greater crystal lattice energy; in one (phenylalanine) the two forms are nearly equal; and in two (glutamic acid and diiodotyrosine) the d,l-form has the lower crystal lattice energy. Generally the form of lower crystal lattice energy is the more soluble of the two at room temperature; but its solubility increases with temperature less rapidly than that of its isomer. For instance, d-alanine is more soluble than d,l-alanine at 0°, but distinctly less soluble at 100° (see Chapter XV, Table IX).

Density also is closely related to crystal lattice energy since high density reflects close packing of molecules in the crystal (77). Thus, for leucine and valine (Table X) the racemic form is denser and shows a larger heat of solution than the optically active form. In the case of glutamic acid, the d-form is denser than the d,l-form and shows also higher heat of solution. For the amino acids thus far studied the greater crystal density is always shown by the form possessing the greater heat of solution.

Among other types of isomers, α -aminoisobutyric acid is markedly denser and also less soluble in water than α -amino-n-butyric acid (Table X). For such isomers as this, ΔH_m can hardly be considered identical for the two forms, but it is probably nearly the same, and the heat of solution of the iso-acid should therefore be larger than that of its n-isomer.

Dalton and Schmidt (54, 55) have discussed the question whether the d,l-forms of the amino acids exist as racemic compounds or racemic mixtures. They conclude that all the substances listed in Table X are racemic compounds with the possible exception of glutamic acid which they consider doubtful. The marked difference in density between the d- and the d-l-forms of glutamic acid suggests that it too exists as a racemic compound rather than as a mixture. Loring and du Vigneaud (78) by studying solubility in the presence of excess d- or l-cystine, have shown that d-cystine is a racemic compound, and the same method should be applicable to other amino acids.

6. Relative Solubility in Water and Organic Solvents

At constant temperature the saturated solution of any substance which forms a pure solid phase must be in equilibrium with the pure solid, and with its saturated vapor at the pressure p° . Hence the vapor pressure, and the activity, of the solute must be the same in all saturated solutions provided that the same solid phase is in equilibrium with all of them. Considering two different solvent media, A, and B, we may therefore write from equation (16)

$$N_A = K_A p^{\circ}$$
 and $N_B = K_B p^{\circ}$ (21)

The activity coefficient, f, is defined as the ratio of activity to mole fraction (a/N). Since a is the same in the two saturated solutions, we may write:

$$a = f_A N_A = f_B N_B \tag{22}$$

and combining equations (21) and (22):

$$\frac{N_A}{N_B} = \frac{K_A}{K_B} = \frac{f_B}{f_A} \tag{23}$$

Thus the activity coefficients f_A and f_B are the reciprocals of the distribution coefficients K_A and K_B .¹⁷

¹⁷ The solubility ratio does not necessarily give the activity coefficient ratio for the solute at infinite dilution in the two solvents A and B. To determine this, we must know how the activity coefficient varies with concentration in each solvent, as determined by freezing point, vapor pressure, or E.M.F. measurements.

Very accurate measurements on the freezing points (79) of glycine solutions, and of their relative vapor pressure at 25° by an isopiestic method (80) are now available. These show that $\gamma(=a/m)$ for a 2.0 molal solution at 0° is 0.733, and for a 3.3 molal solution is 0.729 at 25°. (These solutions are approximately saturated.) In the latter case, $\log \gamma = -0.137$. d- and d, l-alanine, on the other hand, appear from the freezing point measurements of Frankel (81) to behave as nearly ideal solutes; the same is apparently true of valine and leucine. According to Hoskins, Randall and Schmidt (82) aspartic and glutamic acids have very low activity coefficients. All these data and others are more fully discussed in Chapter XII.

Apart from aspartic and glutamic acids, and possibly from the very soluble molecules, proline and hydroxyproline, saturated glycine solution shows a greater deviation from the ideal solution laws than any of the other α -amino acids may be expected to show. (It is not unlikely that other α -amino acids show greater deviation from the ideal solution laws in dilute solution than does glycine; but since they are so much less soluble, γ in a saturated solution is unlikely to deviate so far from unity for other amino acids as for glycine.) The solubility of all amino acids in organic solvents is low, and the activity coefficient may be taken as independent of the concentration in such solvents. Thus the value $\log \gamma = -0.137$ for glycine (saturated) shows the maximum deviation to be expected for α -amino acids from the assumption that the ratio $\log N_A/N_W$ (relative solubility in water and another solvent, A) gives

Table XI

Solubility of Certain Amino Acids and Peptides in Water, Ethanol and Ethanol-Water Mixtures at 25°

	Water	20 per cent Ethanol	40 per cent Ethanol	60 per cent Ethanol	80 per cent Ethanol	90 per cent Ethanol	Ethanol
			Glycine				
Conc. (mole/L)	2.886	1.343	0.507	0.157	0.0278	0.00556	0.00039
Log N	-1.247	-1.545	-1.910	-2.339	-2.975	-3.595	-4.638
			d,l-Alanine	(83, 77)			
Conc.	1.656	0.877	0.402	0.158	0.0359	0.00794	0.00076
Log N	-1.491	-1.728	-1.987	-2.334	-2.863	-3.441	-4.347
			β -Alanin	e (84)			
Conc.	6.123	5.180	3.531	1.687	0.242	0.0382	0.00189
Log N	-0.816	-0.876	-1.011	-1.283	-2.033	-2.759	-3.955
		d,l-a	x-Amino-n-bu	tyric acid (7	7)		
Conc.	1.800	1.082	0.570	0.260	0.0668		0.00260
Log N	-1.440	-1.627	-1.854	-2.116	-2.594		-3.818
		α-2	Amino-iso-but	yric acid (77)		
Cone.	1.330	0.775	0.401	0.177	0.0467	*	
Log N	-1.583	-1.780	-2.009	-2.284	-2.750		
			d-Valin				
Conc.	0.706	0.409	0.231	0.123	0.0373		
Log N	-1.870	-2.063	-2.252	-2.442	-2.848		
			d,l- $Valin$				
Conc.	0.571	0.318	0.167	0.0860	0.0280	0.00922	0.00128
Log N	-1.967	-2.175	-2.395	-2.599	-2.971	-3.376	-4.125
			no-n-caproic			0.075	1.120
Conc.	0.0866	0.0516	0.0346	0.0271	0.0130	0.00585	0.00104
Log N	-2.801	-2.971	-3.083	-3.102	-3.304	-3.573	-4.215
LUG II			l-Leucin		0.001	0.0.0	1.210
Conc.	0.171	0.0977	0.0620	0.0441	0.0204	0.00770	0.00128
Log N	-2.503	-2.695	-2.827	-2.889	-3.109	-3.455	-4.125
Log II		2.000	d.l-Leuci		0.100	0.100	1.120
Conc.	0.0744	0.0423	0.0264	0.0186	0.00848		
Log N	-2.870	-3.059	-3.199	-3.266	-3.491		
1108 11	2.0.0		e-Aminocapro		0.101		
Conc.	3.848	3,439	2.852	1.909	0.485	0.0713	0.00194
Log N	-0.975	-0.998	-1.050	-1.185	-1.719	-2.487	-3.947
Tog 14	-0.515	-0.556	l-Aspartic		-1.715	-2.401	-0.021
Conc.	0.0375	0.0149	0.00675	0.00264	0.00070	0.00021	0.0000116
Log N	-3.168	-3.513	-3.793	-4.114	-4.580	-5.019	-6.167
TOR IA	-5.100	-0.010	l-Asparag		-4.000	-5.015	-0.101
Conc.	0.186	0.0750	0.0306	0.0105			0.000023
	-2.468	-2.810	-3.135	-3.514			-5.870
$\log N$	-4.408	-2.010	—3.135 Diglycii				-3.610
Cons	1,512	0.531		0,0362	0.00374	0.000493	0.0000222
Conc.		-1.948	0.152	-2.977	-3.845	-4.648	-5.889
Log N	-1.522	-1.048	-2.436		-0.040	-4.040	-0.009
0	1 0000		Triglyci	ne (84)	0.000608	0.0000381	0.00000106
Conc.	1.0229						
Log N	-2.241				-4.636	-5.672	-7.206

Relative solubility in two solvents is thus independent of the vapor pressure of the solute and of its crystal lattice energy. It follows that solubility ratios should be more simply related to the chemical structure of the solute than the absolute value of solu-

the relative activity coefficients of the amino acid in the two solvents. For the very soluble β -, γ -, δ -, and ϵ -amino acids of high solubility and high dipole moment, the deviation may well be greater than this; and the same may be true of some of the highly soluble peptides, such as lysylglutamic acid.

Table XI (Continued)

Solubility of Other Amino Acids in Water and Ethanol at 25°

Water Ethanol	Water Ethanol	Water Ethanol
d-Glutamic acid (58) Conc. (mole/L) 0.0585 0.0000185 Log N -2.975 -5.967	d-Glutamine (58) 0.291 0.0000315 -2.269 -5.735	d,!-Serine (55,*) 0.0000639* -2.065 -5.427*
Betaine (85) Conc. Log N -0.69 -1.44	d,l-Threonine (*) (α-amino-β-hydroxybutyric acid) 1.598* 0.000453* -1.507 -4.577*	

Solubility of Amino Acids in Water at 25°1

		Conc.	Log N			Conc.	Log N
d-Alanine ((54)		-1.486	Taurine	(55)		-1.832
d,l-Aspartic acid (54)		-2.978	l-Tryptophane	(55)		-3.000
l-Cystine (55,	78)	0.000450	-5.105				
				l-Tyrosine	(76)		-4.15
d-Cystine (78)	0.000450	-5.105	d-Tyrosine	(76)		-4.15
d,l-Cystine (78)	0.000208	-5.434	d,l-Tyrosine	(76)		-4.46
	(78)	0.000234	-5.376	d,l-Diiodotyrosine	(76)		-4.85
d,l-Glutamic acid (54)		-2.599	l-Diiodotyrosine	(76)		-4.58
				l-Dibromotyrosine	(76)		-3.84
	(86)		-1.326	l-Dichlorotyrosine	(76)		-3.85
The state of the s	54)		-2 .515	(anhydrous)			
d-Isoleucine ((55)		-2.249	d.l-Phenylalanine	(54)		-2.815
				l-Phenylalanine	(55)		-2.492
	55)		-2.393				
l-Proline (86)		-0.693	Sarcosine	(87)	4.81 (20°)	

^{*} Unpublished data from this laboratory. The sample of d,l-threonine studied was supplied by Prof. W. C. Rose.

The following table gives the relation between the volume per cent of ethanol in the solvent as stated in Table XI, the weight per cent ethanol in the solvent, the mole fraction of ethanol, and the dielectric constant at 25°.

Volume per cent ethanol	10	20	30	40	50	60	70	80	90	100
Weight per cent										
ethanol	7.98	16.16	24.54	33.24	42.37	52.03	62.34	73.45	85.65	100
Mole fraction										
ethanol	0.0328	0.0701	0.1129	0.1630	0.2234	0.2979	0.3931	0.5197	0.7001	1.0
Dielectric con-										
stant at 25°	73.89	69.47	64.45	59.16	53.44	47.70	41.51	35.72	29.95	24.28
Solubilities of	several a	mino acio	ls in alco							

¹ For complete solubility equations see Table IX, Chapter XV.

bility in either solvent. The latter is a function of p° , which is in turn determined by the intermolecular forces in the crystal lattice of the pure solute. Relatively slight changes in the chemical groupings within a molecule often cause great alterations in the crystal lattice structure and hence in solubility, while such complications are absent when only solubility ratios are considered. Thus l-leucine is more than twice as soluble as d_{l} -leucine both in water

and 80 per cent ethanol; but the relative solubility of both substances in the two solvents is the same practically within the limits of experimental error (Table XI).

Such solubility ratios should also be ultimately of biological significance. The organization of the living cell certainly involves aqueous phases of high dielectric constant and fatty phases of low dielectric constant. The distribution coefficients of many types of

Table XII

Solubility of Glycine, d.l-a-Amino-n-Caproic Acid and Certain Amino Acid Derivatives
in Various Solvents at 25°

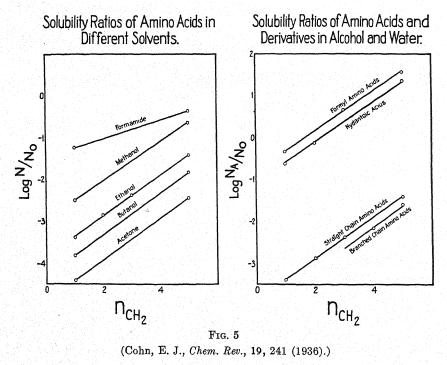
	Conc. log N	Conc. log N	Cone. log N	Conc. log N	
Solvent	Glycine (84)	d,l-α-Amino-n- caproic acid (84)	Hydantoic acid (84)	α-Aminocaproic hydantoic acid (84)	
Water	2.886 -1.247	0.0866 -2.801	0.329 -2.217	0.00690 -3.903	
Formamide	0.0838 -2.476	0.0173 -3.161	0.837 - 1.462	0.165 - 2.175	
Methanol	0.00426 -3.762	0.00854 -3.458	0.0797 -2.488	0.1123 -2.335	
Ethanol	0.00039 -4.638	0.00104 -4.215	0.0242 - 2.851	0.0477 -2.551	
Butanol	0.0000959 -5.055	0.000336 -4.512	0.00643 - 3.228	0.01786 - 2.785	
Acetone	0.0000305 -5.648	0.0000793 -5.233	0.00248 - 3.737	0.00463 -3.466	
	α-Alanine hydantoic acid (84)	β-Alanine hydantoic acid (84)	e-Aminocaproic acid (84)	Diglycine hydan- toic acid (84)	
Water	0.193 -2.451	0.158 -2.539	0.00690 -3.903	0.126 -2.638	
Ethanol	0.0440 -2.590	0.0170 -3.002	0.00756 - 3.354	0.00115 -4.171	
$Butanol.\dots \\$	_		0.00278 -3.593		
	Triglycine hydantoic acid (84)	Formyl- glycine (84)	Formyl-α-amino- butyric acid (84)	Formyl- leucine (58)	
Water	0.0446 -3.092	1.849 -1.432	0.256 -2.325	0.185 -2.469	
Methanol		0.710 -1.533	0.646 -1.539		
Ethanol	0.000077 -5.345	0.295 -1.762	0.355 -1.674	1.792 -0.911	
Heptanol		0.0347 -2.311	0.0500 -2.152		
	Hydantoin (58)	Hydantoin of α-amino- n-butyric acid (58)	Hydantoin of leucine (58)	Aspartic acid hydantoin (58)	
Water	0.397 -2.136	0.863 -1.775	0.0124 -3.650	0.0705 -2.893	
Ethanol	0.0324 -2.721	0.988 -1.254	0.100 -2.229	0.0141 - 3.083	

molecules between different solvents of these two types must play an important part in the chemical dynamics of the cell; and comparison of the relative solubilities of two different molecules in such solvents as water and alcohol will indicate, at least approximately, their relative distribution coefficients between other aqueous solutions and other organic solvents.

The solubilities of many amino acids and closely related substances, in water and organic solvents at 25° are given in Tables XI and XII. Several general correlations between chemical struc-

ture and solubility may be stated and illustrated from the material in these tables.

(1) Effect of the CH₂ Group on Solubility and Activity Coefficients. The solubility in water of the monoaminomonocarboxylic acids, from glycine to leucine and norleucine, decreases with increasing number of CH₂ groups in the hydrocarbon chain. The aminobutyric acids are exceptions to this rule, being more soluble than alanine. Probably this abnormality is due to their crystal



structure and low crystal lattice energy (77). If solubility ratios in water and organic solvents are considered, however, the effect of the CH_2 group is perfectly regular, and may be described by a simple rule. (58) "Roughly stated this is that the ratio of the solubility in alcohol, N_A , to that in water, N_0 , is increased threefold for each CH_2 group in the hydrocarbon chains terminating in methyl groups. This rule appears to hold not only for amino acids but for other sufficiently insoluble compounds, such as the formyl amino acids, hydantoins, and hydantoic acids" (37 p. 119). A general statement of the rule, applicable to several organic solvents, is given in the equation:

where n is the number of the CH₂ groups, and K_2 a constant depending on the nature of the polar group. The coefficient K_1 , defining the influence of CH₂ groups upon the solubility ratio, varies, of course, with the pure solvents, being 0.44 for methanol, 0.49 for ethanol and acetone, and 0.53 for butanol and heptanol (84). The logarithm of the solubility ratio for several homologous series and several solvents is plotted as a function of the number of CH₂ groups in Fig. 5. Similar results have been obtained by a study of the distribution coefficients of amino acids between water and butanol (89).

This effect of hydrocarbon residues on the solubility ratio reflects the same forces that give rise to the orientation of molecules in surface films (90). Long-chain fatty acids or alcohols are oriented at an interface so that the polar -OH or -COOH group dips into the water, while the non-polar hydrocarbon chain is repelled from it. The accumulation of non-polar groupings at the interface lowers the surface tension of the water. In 1891 Traube (91) stated the rule that with molecules of aliphatic compounds having different lengths of hydrocarbon chains, the decrease in the surface tension of the pure liquid, divided by the partial osmotic pressure of the dissolved substance in the underlying solution, for dilute solutions increases about threefold for each CH2 group. Recently Pappenheimer, Lepie, and Wyman (92) have shown a similar effect of the CH2 group on the surface tension of amino acid solutions. The effect of the CH2 group on vapor pressures and distribution coefficients in the various homologous series shown in Table VIII obviously reflects the same influences.

The rule as stated holds only for CH₂ groups in hydrocarbon chains terminating in methyl groups. Thus $\log N_A/N_o$ is virtually identical for asparagine and glutamine, and likewise for aspartic and glutamic acids (Table XI). These two pairs of compounds differ only by a CH₂ group which is "shielded" by a COOH group at the end of the chain, whereby its power of influencing the solubility ratio is apparently annulled (58, 84, 93). In other cases, as in ϵ -aminocaproic acid, CH₂ groups between polar groups appear to exert some effect in enhancing the solubility ratio, but not the full effect exerted by a side chain of the same length. The problems raised by these observations have been discussed in detail by McMeekin, Cohn and Weare (84).

(2) The Effect of Various Other Groups on Solubility and Activity Coefficients. The benzene ring, like an aliphatic hydro-

carbon radical, may be expected to increase solubility in alcohol relative to that in water. This expectation is confirmed by a comparison of alanine and phenylalanine (Table XIII), the relative solubility of the latter in ethanol as compared to water being about 28 times as great as for alanine. On this basis, the benzene ring has approximately the same effect as three CH₂ groups; but further measurements are needed to determine how generally this relationship holds.

The polar hydroxyl group has the opposite effect. Comparison of

Table XIII

Influence of Various Groups on the Solubility Ratio in Ethanol and Water

(All data are at 25°)

Substance	$\log N_A/N_0$	Substance	$\log N_A/N_0$	Effect of Substituent $\Delta \log N_A/N_0$
	Influenc	e of Benzene Ring		
d,l-Alanine	-2.856	d,l-Phenylalanine	-1.453	+1.403
	Influence	of Hydroxyl Grou	p	
d,l -Alanine d,l - α -Amino- n -butyric	-2.856	d,l-Serine	-3.362	-0.506
acid	-2.375	d, l -Threonine	-3.070	-0.695
	Influ	ience of CONH		
Glycolamide	-0.799	Hydantoic acid	-0.630	+0.169

alanine and serine, and of α -amino-n-butyric acid with threonine (Table XIII) shows that substitution of a hydroxyl group for a hydrogen reduces N_A/N_0 between three and five fold.

The effect of the -CONH- group is more complicated, depending on its position in the molecule. When situated between polar groups, it very markedly diminishes this ratio, as a comparison of the glycine peptides and of their hydantoic acids definitely indicates. The substitution of a -CONH₂ group for a hydrogen atom at the end of a chain, however, appears to produce only a very slight effect on the solubility ratio in compounds possessing other polar or charged groups. The comparison of hydantoic acid (HOOC·CH₂·NH·CONH₂) and glycolamide (HO·CH₂·CONH₂), whose net structural difference amounts to one -CONH- group, indicates a change in log N_4/N_0 of only +0.169 due to this dif-

ference; and study of other related compounds containing a terminal -CONH₂ group indicates that its effect on the solubility ratio is generally as small or smaller than this (84).

(3) Influence of Dipolar Ionization on Solubility and Activity Coefficients. Dipolar ions are far more soluble in water, relative to organic solvents, than their uncharged isomers. This is shown most directly (Table XIV) by a comparison of simple amino acids with the isomeric hydroxy-amides, with which they have already been compared in evaluating the electrostriction effect (Table V).

¹⁸ The relative activity coefficients of dipolar ions and uncharged molecules in different media have also been evaluated by a method quite independent of solubility measurements. As shown in Section I, the ratio of concentrations (or mole fractions) of dipolar ions and uncharged molecules in water (K_Z) is approximately determined by the first dissociation constant of the amino acid in water K_1 , and that of its ester, K_E .

$$\log K_Z = \log \left(\frac{a_R \pm}{a_R}\right) = \log \left(\frac{N_R \pm}{N_R}\right)_{\text{H}_2\text{O}} = pK_B - pK_1$$
 (12a)

where the subscript R^{\pm} refers to the dipolar ion, $H_3\tilde{N} \cdot R \cdot COO$, and R to its uncharged isomer, $H_2N \cdot R \cdot COOH$. In another medium (B), the corresponding pK values may be denoted by pK_E^* and pK_1^* . In the medium B, then:

$$\log\left(\frac{N_R \pm}{N_R}\right)_R = pK_E^* - pK_1^* \tag{12b}$$

But by definition the activity ratio $a_R \pm /a_R$ of the two forms must be a constant at a given temperature and pressure. In any medium (see equations (22) and (23)) $a_R \pm = f_R \pm N_R \pm$, and $a_R = f_R N_R$, where the activity coefficients $f_R \pm$ and f_R are taken as unity in water. Hence in the medium B:

$$\left(\frac{f_R \pm}{f_R}\right)_B \times \left(\frac{N_R \pm}{N_R}\right)_B = \left(\frac{a_R \pm}{a_R}\right) = K_Z \tag{12e}$$

Whence, combining equations (12a), (12b), and (12c), the logarithm of the activity coefficient ratio is given by the equation:

$$\log\left(\frac{f_R\pm}{f_R}\right)_B = (pK_E - pK_1) - (pK_E^* - pK_1^*). \tag{12d}$$

By measurement of relative dissociation constants in water and 90 per cent ethanol, Edsall and Blanchard (3) concluded that $\log (f_R \pm /f_R)$ lies in the range 2.3 to 2.7 for several amino acids in this solvent. This is quite close to the values derived from solubility measurements in water and absolute ethanol and recorded in Table XIV. The extrapolation to absolute ethanol of the results derived from dissociation constant measurements, therefore, yields results somewhat higher than those from solubility. Very accurate determinations of dissociation constants in media other than water (for instance by concentration cells without liquid junction) should lead to more exact knowledge of the activity coefficients as determined by equation (12d). These will not probably in any case agree exactly with values derived from solubility measurements, since different types of uncharged isomers are being considered in the two cases.

The amino acids are approximately 400 times as soluble in water relative to ethanol, as the hydroxy-amides.

A similar comparison may be made between amino acids and peptides, on the one hand, and the corresponding hydantoic acids on the other (84). Each dipolar ion differs by a terminal -CONH-group from its hydantoic acid, but it has already been pointed out that the effect of this group on the solubility ratio is apparently

TABLE XIV

Influence of Dipolar Ionization on Relative Solubility

(All data are at 25°)

Organic solvent	Substance	Log of solubility ratio log N/N.	Substance	Log of solubility ratio log N/N:	Influence of dipolar ionization $\Delta \log N/N$
Es Ethanol	timated by Comparison	of Amino	Acids and Hydroxyamides in Wate	r and Ethar	-2.592

Formamide	Glycine α-Aminocaproic acid	-1.229 -0.360	Hydantoic acid α-Aminocaproic hydantoic acid	$+0.755 \\ +1.728$	-1.98 -2.09
Methanol	Glycine	-2.515 -0.657	Hydantoic acid α-Aminocaproic hydantoic acid	-0.271 + 1.568	-2.24 -2.23
Ethanol	Glycine	-3.391	Hydantoic acid	-0.630	-2.76
	α-Alanine	-2.856	α-Alanine hydantoic acid	-0.139	-2.72
	β-Alanine	-3.139	β-Alanine hydantoic acid	-0.463	-2.68
	α-Aminocaproic acid	-1.414	α-Aminocaproic hydantoic acid	+1.352	-2.77
	Diglycine	-4.367	Diglycine hydantoic acid	-1.533	-2.83
	Triglycine	-4.965	Triglycine hydantoic acid	-2.253	-2.71
Butanol	Glycine	-3.808	Hydantoic acid	-1.011	-2.80
	α-Aminocaproic acid	-1.711	α-Aminocaproic hydantoic acid	+1.118	-2.83
Acetone	Glycine	-4.401	Hydantoic acid	-1.520	-2.88
	α-Aminocaproic acid	-2.432	α-Aminocaproic hydantoic acid	+0.437	-2.87

small (+0.17 or less in its effect on $\log N_A/N_0$), and furthermore it will presumably be essentially the same throughout the whole series.

The influence of dipolar ionization is on the whole remarkably constant in each solvent. The extensive data for ethanol show that it is independent of the dipole moment of the dipolar ion, even for substances differing as widely in moment as glycine and triglycine. This could not be true if the observed differences were determined entirely by electrostatic forces; for in this case the effect of dipolar

ionization should be greatest for the substances of greatest dipole moment. The same conclusion is reached by a study of the results in formamide, which has a dielectric constant greater than water (>84). On purely electrostatic grounds this should result in a higher value of $\log N/N_0$ for dipolar ions than for uncharged com-

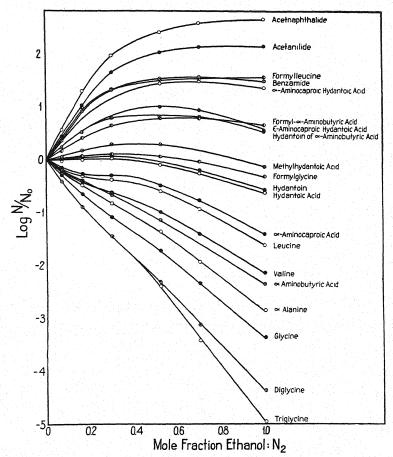


Fig. 6. Solubility of amino acids and other molecules in ethanol-water mixtures. The solubility in water (N_0) is taken as the standard of reference for each substance. (Cohn, E. J., Chem. Rev., 19, 241 (1936).)

pounds in such a solvent. The opposite is in fact the case; the effect of dipolar ionization is the same in direction, although smaller in magnitude, than in the solvents of very low dielectric constant. Evidently the differences between the two classes of compounds are determined in large measure by non-electrostatic forces. Specific interactions between the different chemical groups in both

solvents and solutes must clearly be taken into account in addition to the electrostatic forces.

(4) Solubility in Ethanol-Water Mixtures. The solubility of a group of charged and uncharged organic molecules in alcohol-

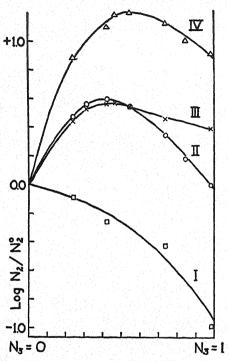


Fig. 7. The relationship between the logarithm of the solubility ratio and the mole fraction of ethanol in the alcohol-water mixture for l-tyrosine (Curve I), l-dibromotyrosine (Curve II), l-dichlorotyrosine (Curve III), and l-dichlorotyrosine (Curve IV). Maximum error in data: l-tyrosine and l-dichlorotyrosine ± 5 per cent; l-dibromo- and l-dichlorotyrosine ± 1.6 per cent.

(Winnek, P. S., and Schmidt, C. L. A., J. Gen. Physiol., 19, 773 (1936).)

water mixtures is shown graphically in Figs. 6 and 7. At one extreme in Fig. 6 is acetnaphthalide, with a polar residue attached to the very large non-polar naphthalene ring; at the other is triglycine, with a very high dipole moment and two peptide linkages; and the intervening series of molecules forms a progressive series between the two extremes. All the molecules shown are fairly polar; a truly non-polar molecule, like hexane, would doubtless show a curve rising far more steeply even than that of acetnaphthalide.

Notable is a group of compounds which are more soluble in ethanol-water mixtures than in pure water or ethanol; such sub-

stances as the dihalogenated tyrosines (94), methylhydantoic acid, ϵ -aminocaproic hydantoic acid, and formylglycine. All these molecules contain uncharged polar groups; the dihalogenated tyrosines, which are dipolar ions, contain also the benzene ring with its phenolic groups and two halogen atoms. Apparently molecules of this sort exert attractive forces on both water and alcohol molecules which are more fully satisfied in a mixture of these two solvents than in either one alone. The same type of behavior, in a more extreme form, is found among a class of proteins, the prolamins (see Section III).

7. Solubility of Amino Acids in Solutions of Salts and Other Amino Acids

The presence of dissolved salts exerts an important influence on the solubility of amino acids. Likewise salts exert important solvent or precipitating action on other slightly soluble salts or on uncharged organic molecules. The nature of these two latter effects may be briefly stated here, since they form a preliminary basis for the understanding of the behavior of dipolar ions in salt solutions.

Since the activity of the solute must be the same in all saturated solutions, its relative solubility in any two solvents equals the reciprocal of the activity coefficient ratio in the two solutions (equations 23).¹⁹ If the activity coefficient, γ , is defined as unity for zero salt concentration, where the solubility is S', then in any other saturated solution

$$\log S/S' = -\log \gamma$$

by definition.

(1) Interaction of Salts in Dilute Solution. In sufficiently dilute solution, slightly soluble salts become more soluble in the presence of other salts. Salts containing bi- or trivalent ions are found to exert much greater solvent action than uni-univalent electrolytes. The effect of each ion is proportional to the square of its valence, and to describe the phenomenon Lewis and Randall (52, p. 373) introduced a new term, the ionic strength μ :

$$\mu = 1/2 \sum m_i Z_i^2 \tag{25}$$

¹⁹ The activity coefficient γ denotes the ratio a/c or a/m; f denotes a/N. In dilute solution the ratio of two solubilities will be virtually identical whether they are expressed as moles per liter, S, or mole fraction, N. In such cases, therefore, γ and f may be regarded as identical.

that is, half the sum taken over all the ions in solution, of the molality of each ion times the square of its valence. The factor $\frac{1}{2}$ is introduced to make the ionic strength equal to the molality for a uni-univalent salt. For uni-bivalent salts (as BaCl₂ or NaSO₄) the ionic strength is three times the molality; for bi-bivalent salts (as MgSO₄) it is four times as great. The ionic strength, in moles per liter, is denoted by $\Gamma/2$:

$$\Gamma/2 = 1/2 \sum c_i Z_i^2 \tag{26}$$

Lewis and Randall formulated the rule: in dilute solutions, the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength.

Debye and Hückel (95; see also 96 and 97) showed that this rule could be explained in terms of the Coulomb forces (equation 13) between the ions in solution. They deduced a general equation, applicable to very dilute ionic solutions, relating the activity coefficient of a salt to the total ionic strength of the solution. For a salt dissociating into two kinds of ions, this reads:

$$-\log \gamma = \log \frac{S}{S'} = \frac{1.81 \times 10^6}{D^{3/2} T^{3/2}} Z_1 Z_2 \sqrt{\frac{\Gamma}{2}}$$
 (27)

Here D is the dielectric constant of the solvent, T the absolute temperature, Z_1 and Z_2 the valences of the two ions of the salt. For water at 25° (D=78.54) this reduces to

$$-\log \gamma = 0.505 Z_1 Z_2 \sqrt{\Gamma/2} \tag{28}$$

In biochemical systems, however, the dielectric constant often deviates widely from that of water, owing to the presence either of dipolar ions which raise the dielectric constant or of fatty substances which lower it. In such cases the more complete equation (27) must be employed.

According to this equation then, the logarithm of the activity coefficient or of the relative solubility is proportional (a) to the square root of the ionic strength, (b) inversely to the three-half power of the dielectric constant, (c) to the product of the valence of the ions of the dissolved salt. The standard state (solubility S') is to be taken as the same solvent, but without salt, that is at zero ionic strength.

These relations hold accurately only as limiting laws in very dilute salt solutions. In more concentrated solutions, correction

terms must be introduced for the finite radii of the ions, and for the effects of attraction between the ions and the solvent dipoles. These are discussed in detail elsewhere (95, 97), and will not be treated here.

(2) Interaction of Salts and Uncharged Molecules. While salts tend to dissolve other slightly soluble salts, they decrease the solubility of most uncharged molecules. This, the well-known "salting-out effect," probably arises from the tendency of polar molecules to cluster around the salt ions, due to the intense inhomogeneous field in the neighborhood of each ion; a tendency already discussed in connection with the electrostriction of the solvent. Since most uncharged molecules have smaller dipole moments per unit volume than water, the water is attracted about the ions, thereby, in effect, repelling the other molecules, whose solubility is thus diminished. In a great number of systems of this sort, it has been found that the logarithm of the solubility of the uncharged molecule is directly proportional to the first power of the concentration of the added salt:

$$\log S/S' = K_s C \tag{29}$$

Here S' is the solubility in the absence of salt, C the salt concentration, and K_s , the salting-out constant, depends in general on the nature of the salt and on the effect of the solute on the dielectric constant of water. For most substances K_s is positive (log S/S' negative); but a few substances such as urea and hydrocyanic acid, which raise the dielectric constant of water, become more soluble in the presence of salts.²⁰

The value of K_s differs greatly for different salts acting on the same uncharged molecule; salts with bivalent or trivalent anions, such as the carbonates, sulfates, phosphates, and citrates, have a much larger salting-out effect than salts of bivalent cations, such as calcium or zinc chlorides. These relations appear to be very general, and as a first approximation the relative K_s -values for various salts are nearly independent of the particular molecule being salted out.

(3) Interaction of Salts and Amino Acids. Since amino acids carry ionic charges, they are closely akin to salts; since they possess CH₂ groups and other organic residues, they must behave in many

²⁰ Concerning the salting-out effect see Debye and McAulay (98), Scatchard (99), and Debye (100). Falkenhagen (97) gives a comprehensive discussion with numerous references.

respects like other organic molecules. Thus the presence of neutral salts might tend either to dissolve or to precipitate amino acids. For the pioneer investigations in this field we are indebted to Pfeiffer and his collaborators (101, 87), and the results of some

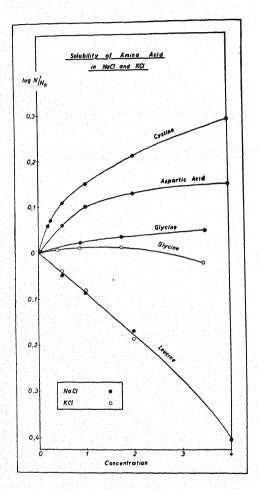


Fig. 8. The data for cystine are taken from unpublished data by McMeekin and Cohn. The other data are taken from the paper by Pfeiffer and Würgler (101).

(Cohn, E. J., *Naturwiss.*, 20, 673 (1932).)

of their experiments, with those of other workers (102), are plotted in Figs. 8 and 9. Glycine, cystine, and aspartic acid, which carry large electric charges and small hydrocarbon residues, are dissolved by neutral salts, while leucine, with its long hydrocarbon chain, is salted out by sodium and potassium chlorides. The work

of Euler and Rudberg (104) shows an even greater salting-out effect for tyrosine by sodium chloride. The solvent action of calcium chloride is much greater than that of sodium or potassium chlorides; even leucine, which is salted out by the two latter salts, is dissolved by calcium chloride.

In solvents of low dielectric constant, electrostatic forces are

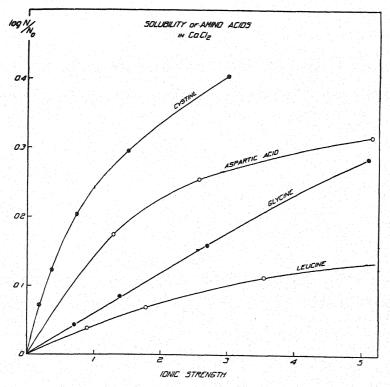


Fig. 9. The data for cystine are taken from the paper by Blix (102). The other data are reported by Pfeiffer and Würgler (101).

magnified. Under these circumstances, the solubility of amino acids in the absence of salts is very small, but the solvent power of salts becomes much greater than in water (Fig. 10); even leucine, which is salted out by sodium chloride in water, is dissolved by lithium chloride in 90 per cent ethanol to approximately the same extent as glycine (Fig. 11). The solvent action on β -alanine, with its higher dipole moment, is greater than on either α -amino acid. The influence of calcium chloride, per mole of salt, is approximately three times as great as that of lithium chloride; which sug-

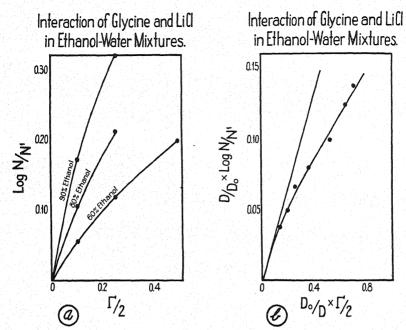


Fig. 10. Fig. 10a shows the increasing solvent action of lithium chloride on glycine with decrease in the dielectric constant of the solvent. All of the data in Fig. 10a fall on one curve when plotted as in Fig. 10b (Equation (32)).

(Cohn, E. J., Chem. Rev., 19, 241 (1936).)

Solubility of Amino Acids in 90% Alcohol containing LiCl

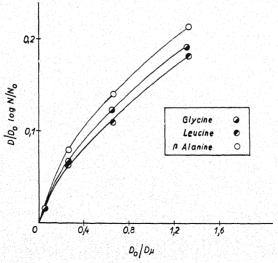


Fig. 11 (Cohn, E. J., Naturwiss., 20, 663 (1932).)

gests that the principle of the ionic strength obtains also in these systems, and that the phenomena may be interpretable in terms of Coulomb forces.

Scatchard and Kirkwood (105) extended Debye's theory of interionic attraction to the case of dipolar ions. They concluded that the activity coefficient of a dipolar ion should be decreased in the presence of a neutral salt; $-\log \gamma$ being directly proportional

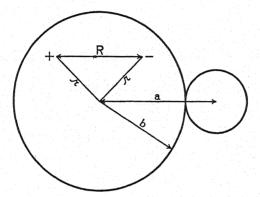


Fig. 12. Kirkwood's model of a dipolar ion containing a single positive and a single negative charge. The large circle of radius b represents the spherical dipolar ion; the small circle represents an ion. The distance a is the closest distance of approach between the centers of the ion and the dipolar ion. The positive and negative charges in the dipolar ion are separated by the dipole distance R, and r is the distance of each charge from the center of the dipolar ion.

to the ionic strength instead of its square root, and inversely proportional to the square of the dielectric constant of the solvent. These relations, of course, hold only as a limiting law at very low ionic strengths; and it is assumed furthermore that the dipolar ion is present in a concentration too low to affect the dielectric constant of the solvent appreciably. Subject to these limitations, these conclusions of Scatchard and Kirkwood have been confirmed by later theoretical calculations and by all experimental work at present available. Kirkwood (41) has since developed the theory further, and has calculated the effect of salts on a model which appears to give a very good approximation to the behavior of certain dipolar ions. The molecule is considered as a sphere, of radius b, immersed in a continuous solvent of dielectric constant D, which contains free salt ions at ionic strength $\Gamma/2$. "If the mean distance of closest approach of these ions to the complex (dipolar) ion, is a, there will exist a second spherical boundary of radius a, concentric with the sphere b, within which no ions penetrate" (41, p. 352). Within the sphere b are located M discrete point charges, half positive and half negative. For amino acids, one positive and one negative charge, separated by a distance R, will suffice to describe the system; the center of this dipole, however, is not in general located at the center of the sphere but separated from it by a distance r. Kirkwood has given a very general treatment, applicable to all systems of this type. In the case of glycine, however, the center of the dipole must coincide very nearly with the center of the molecule (R=2r); furthermore glycine may be treated as approximately spherical. Under these special conditions, and at very low ionic strengths, Kirkwood's equation becomes:

$$-\log \gamma = \log \frac{S}{S'} = \frac{68.4 \times 10^6}{D^2 T^2} \frac{R^2}{a} \frac{\Gamma}{2}$$
 (30)

when the distances R and a are expressed in Ångström units. As in the earlier treatment of Scatchard and Kirkwood, $-\log \gamma$ is proportional to $\Gamma/2$ and to $1/D^2$. Furthermore it is proportional to the square of the dipole distance R divided by the "collision radius," a, of the molecule and the salt ions. This factor R^2/a for spherical dipolar ions is analogous to the factor Z_1Z_2 in the Debye-Hückel equation for ions.

For solutions at 25° equation (30) may be rewritten, employing the dielectric constant of water at this temperature $(D_0 = 78.54)$ as a standard of reference,

$$\log \frac{S}{S'} = 0.125 \frac{R^2}{a} \left(\frac{D_0}{D}\right)^2 \frac{\Gamma}{2}$$
 (31)

which, on multiplying through by D/D_o becomes

$$\frac{D}{D_0} \log \frac{S}{S'} = 0.125 \frac{R^2}{a} \left(\frac{D_0}{D}\right) \frac{\Gamma}{2}$$
 (32)

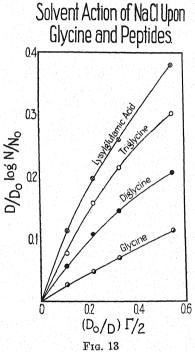
It has proved convenient in practice²¹ to plot $(D/D_0) \log S/S'$ against $(D_0/D) \times (\Gamma/2)$. The limiting slope of this curve, which may be denoted by K_R' ,

$$\lim_{\Gamma/2 \to 0} \frac{(D/D_0) \log S/S'}{(D_0/D)(\Gamma/2)} = K_R'$$
 (33)

²¹ $(D_0/D)(\Gamma/2)$ is directly proportional to the square of the reciprocal length κ , which plays a fundamental part in the Debye-Hückel theory of interionic forces (95, 97).

should on the basis of the theories of Scatchard and Kirkwood be independent of the dielectric constant of the solvent if the observed effects are entirely determined by Coulomb forces. Glycine in ethanol-water mixtures from 60 to 95 per cent ethanol essentially fulfills this condition (Fig. 10). The value of K_{R} is found, within the limits of experimental error, to be the same in all these solvents, its value being 0.32.

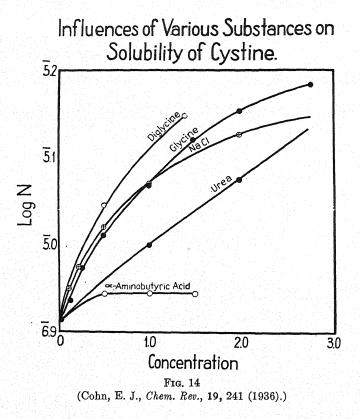
Assuming the simplified model of glycine already discussed, K_{R}'



(Cohn, E. J., McMeekin, T. L., Greenstein, J. P., and Weare, J. H., J. Amer. Chem. Soc., 58, 2365 (1936).)

should be equal to $0.125 R^2/a$. The value of a (Fig. 12) should be equal to the radius of the glycine molecule (b) plus the mean radius of the ions of the salt. This latter quantity has been determined by Pauling (106) as 1.08 Å for lithium chloride. The glycine radius, b, may be estimated from measurements of its apparent molal volume in water (corrected for the shrinkage of the solvent caused by electrostriction) as 2.82 Å. Therefore, a is 3.90 Å; and employing the value 0.32 for K_{R} we obtain 3.15 Å as the value for R, the dipole distance. This is in very good agreement with the value 3.0 Å deduced from a steric model (see Section I, especially Fig. 3).

The effect of salt on the solubility of glycine and a series of peptides in 80 per cent ethanol is shown in Fig. 13. The solvent action of the salt is much greater for the larger peptides than for glycine, $K_{R'}$ increasing to 0.58 for diglycine, 0.77 for triglycine, and 1.16 for lysylglutamic acid. The order of these substances, as determined from the effect of salt on solubility, is the same as their order



in terms of increasing dielectric increments (Table IV). Indeed, Cohn, McMeekin, Greenstein, and Weare (107) concluded from their analysis of the data that $K_{R'}$ is "as a first approximation proportional to the dipole moment" (see also 93). This simple relation would not be expected to hold for spherical dipolar ions, from Kirkwood's theory. These molecules, however, are decidedly asymmetrical, and can be more truly represented as ellipsoids than as spheres. Kirkwood, in unpublished studies, has calculated the effect of salts on ellipsoidal dipolar ions, and has found that the limiting slope should be nearly proportional to the distance, R,

between the charges of the dipole. Here again, therefore, experiment and theory appear to be in accord.

Fig. 10 shows that in solvents of low dielectric constant $K_{R'}$ is very nearly independent of D. Comparable studies in solvents of very high dielectric constant may be made by employing a solution of a very soluble dipolar ion in water as the solvent, and a very in-

Influence of Amino Acids on Interaction Between NaCl and Cystine.

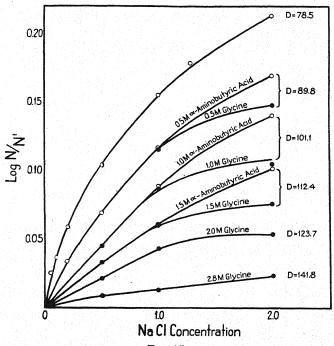


Fig. 15 (Cohn, E. J., Chem. Rev., 19, 241 (1936).)

soluble amino acid as the saturating body. Thus cystine has been studied (93) in aqueous solutions of glycine, α -aminobutyric acid, and also of urea. All of these substances increase the solubility of cystine (Fig. 14). The solvent effect of α -aminobutyric acid, with its longer hydrocarbon chain, is markedly less than that of glycine. Urea has far more solvent action, in proportion to its dielectric increment, than either of the amino acids, suggesting that its mode of action on cystine is somewhat different.

Cystine is thus much more soluble in a concentrated glycine solution than in water; but the effect of salt in increasing the solubility is far less in the solvent of higher dielectric constant (Fig. 15). The results are thus qualitatively in accord with the theory based on Coulomb forces. On closer examination, however, it is found that K_R is not in this case independent of the dielectric

Table XV Values of $(-\delta \ Log \ \gamma_2/\delta\Gamma/2)$ in Dilute Solution

	Molality of elec- trolyte	Leucine	Valine	α-Amino- butyric acid	Alanine	Glycine
NaCl	0.01	-0.09*				0.24†‡
TlCl†	0.005		0.06	0.11	0.15	0.22# 0.30
CaCl ₂	0.01	0.04*				0.30
$\mathrm{ZnCl_2}\dagger$	0.01	0.14 0.14	0.14 0.14	0.15 0.15	0.25 0.26	0.32 0.33

Unless otherwise stated the above measurements were made by Joseph (108). Figures which are italicized refer to a temperature of $0^{\circ} \pm 0.2^{\circ}$; other figures to a temperature of 25°.

* Amino acid solubility, Pfeiffer and Würgler (101).

† E.M.F. measurements (Joseph).

‡ Freezing point measurements, Scatchard and Prentiss (79).

Failey's (110) value from TlCl solubility is 0.32 for saturated TlCl at 25°; this agrees with unpublished results of Straup-Cope and Cohn.

|| The value 0.30 is estimated from freezing points of Pfeiffer and Angern (111) by comparison with freezing point effects calculated for zinc chloride (108). It is significant only to about ± 0.05 , and characterizes not only CaCl₂, but also many other salts of the same valence type.

constant of the solvent, but decreases as D increases. Thus in such systems, other forces than those expressed by Coulomb's law must be operative in the interaction between amino acids and salts. These forces tend to diminish K_{R} , and to produce a salting-out effect, which becomes especially important in solvents of high dielectric constant.

The salting-out effect has also been clearly revealed by the studies of Joseph (108), who has determined salt activities in solutions containing amino acids, from E.M.F. measurements in cells without liquid junction. Employing a relation derived by Bjerrum

(109), relating the activity coefficients of the various components, he calculated the effect of the salt on the activity coefficient of the amino acid, $(-d \log \gamma)/(d\Gamma/2)$. This quantity is virtually identical with K_R . The table compiled by Joseph, from his own observations and those of other investigators using different methods, is reproduced here (Table XV).

For glycine in solutions of calcium chloride and zinc chloride, in water, K_{R}' is virtually the same (0.32) as in solvents of much lower dielectric constant (Fig. 10). This corresponds to the very small salting-out effects generally produced by halides of bivalent cations. The value of K_{R}' for glycine in NaCl, however, falls from 0.32 in 60 to 95 per cent ethanol to 0.24 in water, indicating a well marked salting-out effect.

For a given salt, the value of K_R in water decreases progressively in the series from glycine to leucine, corresponding to the fact that the presence of large hydrocarbon residues always increases the tendency of a substance to be salted out. For leucine in sodium chloride the salting-out term is the predominant factor.

Both the "salting-in" and the "salting-out" effects for dipolar ions are proportional to the first power of the ionic strength (equations 29 and 30 to 32); hence neither can be expected to vanish at infinite dilution.²² Recent studies by Cohn and his collaborators indicate that, as would be expected from calculations of Kirkwood (briefly discussed in (37)), the two effects in combination may be described by an equation of the form

$$\frac{D}{D_0} \log \frac{N}{N'} = \left(K_R - \frac{D}{D_0} K_S \right) \frac{D_0}{D} \frac{\Gamma}{2} = K_{R'} \frac{D_0}{D} \frac{\Gamma}{2}$$
(34)

which is valid in very dilute solutions. The coefficient K_R is, according to Kirkwood's theory, determined by Coulomb forces (41); it depends on the dipole moment and distribution of charge in the dipolar ion, and the radius of the salt ions. K_S , on the other hand, depends on more specific characteristics, both of the salt and of the molecule being salted out. In solvents of low dielectric constant the factor D/D_0 multiplying K_S becomes very small, and

²² This fact emphasizes a fundamental difference between ions, on the one hand, and dipolar ions or uncharged molecules on the other. Ions, in very dilute solution, obey a law of the form: $-\log \gamma = K\sqrt{C}$. Any salting-out term that may be involved is proportional to C; hence, as C approaches zero, \sqrt{C} becomes very large in proportion to C, and the salting-out term for an ion must become negligible in sufficiently dilute solution. This is never true for a dipolar ion or an uncharged molecule.

under these circumstances K_R' becomes nearly identical with K_R . Thus the specific effects of dilute salts on amino acids are nearly eliminated in solvents of low dielectric constant, and the results may be described in terms of the general principle of the ionic strength. In water and solvents of higher dielectric constant, however, the specific influences of individual salts are important even in very dilute solution. Hence the study of such solutions, so important for biology, must involve a detailed knowledge of the nature of the salting-out effect. Such knowledge is still far too meager. The widespread occurrence of the phenomenon, which is found in many systems of very diverse nature, indicates its fundamental importance; further research should do much to clarify and generalize our understanding of its underlying causes.

SECTION III. THE SOLUBILITY OF PROTEINS

8. Certain General Considerations

Different proteins differ so profoundly in their solubility that this property has been commonly employed as a basis for their classification. Thus albumins are readily soluble in water, dilute salt solutions, acids, and alkalies; globulins are insoluble in water but soluble in salts, acids, and alkalies. Prolamins, such as gliadin and zein, are readily soluble in ethanol-water mixtures, but insoluble either in pure water or pure ethanol (112, 113). Such proteins as the keratins or silk fibroin are insoluble in most solvents, except strong acids or alkalies, which must markedly alter their chemical structure.

There is much in these phenomena which recalls similar behavior among the amino acids and their derivatives. Thus the crystalline component of silk fibroin appears to be made up largely of a long-chain polypeptide containing glycyl and alanyl residues (71) with free amino or carboxyl groups only at the extreme ends of the chain. In relative inertness and extreme insolubility, as well as in structure, it markedly resembles the higher peptides of glycine.

Similarly the prolamins resemble substances like the hydantoins and formyl compounds of α -amino-n-butyric acid and of leucine (Fig. 6) and the dihalogenated tyrosines, (Fig. 7), which are more soluble in ethanol-water mixtures than in pure water or pure ethanol. The prolamins, which exhibit the same type of behavior in an extreme degree, possess relatively few free acid and basic groups in proportion to their size. Although they are rich in as-

partic and glutamic acid residues, the carboxyl groups are, for the most part, combined with ammonia to form amide groups (112, 114). The large number of uncharged polar groups on these molecules, and their few charged groups, endow them with affinities for water and alcohols which neither pure solvent alone can apparently satisfy. Some other proteins, besides the prolamins already mentioned, are apparently soluble without denaturation in ethanolwater mixtures. Insulin is soluble in acid solution in 60 per cent ethanol, but very insoluble in 95 per cent ethanol (115, 116). Linderström-Lang (117) has extracted from casein a fraction—shown by Svedberg (118) to be homogeneous with respect to molecular weight—which is readily soluble in warm slightly acidified 60 per cent ethanol.

Most albumins and globulins, like most amino acids and peptides, are very insoluble in organic solvents. In their high solubility in water, and their ready precipitability by organic solvents, the albumins resemble certain highly polar peptides carrying multiple charges, such as lysylglutamic acid. On the other hand, cystine. with its four charges compactly grouped together, shows behavior not unlike that of the globulins, being very insoluble in pure water and becoming much more soluble in the presence of salt. The higher peptides of glycine are somewhat similar. Albumins and globulins may be precipitated from water without denaturation by the addition of ethanol at low temperatures, as was first shown for the serum proteins by Hardy and Gardiner (119). This type of procedure has been employed (120, 121, 122, 123) in recent years for the preparation and purification of several proteins. Exactly similar methods have of course long been used in the preparation of amino acids.

Qualitatively, then, the solubility of albumins and globulins appears to be determined by the same forces already considered in the study of amino acids and peptides²³ (Section II). The magnitude of the effects, however, is far greater in the proteins, as might be expected from their great size and high electric charge. That isoelectric proteins exist as highly charged dipolar ions was shown by Weber (59), who studied the volume changes accompanying

²³ The phenomenon of denaturation (Chapter IX, Section I) forms at present an exception to this general statement. It appears to be intimately related to the complex pattern of the large protein molecule and no close analogy to it in simpler substances has yet been recognized.

ionization in acid and alkaline solution. The ionization of carboxyl and of amino groups shows characteristic differences in the accompanying volume changes. Weber thus showed that, in general, the carboxyl groups in proteins dissociate on the acid side of the isoelectric point, the amino groups on the alkaline side. Even more direct proof of dipolar ion structure is afforded by dielectric constant measurements on isoelectric protein solutions (Section I) which have shown very great dielectric constant increments and, therefore, extremely high dipole moments in proteins. The large radii of protein molecules would permit even a single pair of positive and negative charges at opposite ends of the molecule to give rise to a very large dipole moment. The existence of many positive and negative charges renders the possible dipole moment still greater.

Thus it is not surprising that while the effects of salts on the solubility of amino acids and peptides are comparatively small, and remained practically unrecognized until very recently, the corresponding effects of salts on proteins—their solvent action in dilute solution and their precipitating action in concentrated solution—have been known for nearly a century. The salting out of proteins by concentrated solutions of neutral salts was observed at least as early as 1852 by Panum (124), Virchow (125), and Claude Bernard (126), who made use of the process for the separation of protein fractions. In 1888, Hofmeister (127) studied the salting out of proteins in detail, and formulated rules describing the relative effectiveness of various anions and cations in the precipitation process. Likewise, Panum (128) in 1851 showed that a protein (globulin) could be separated from serum by dilution with water and acidification. This protein was readily dissolved by small amounts of neutral salt. The proteins of blood plasma were intensively studied by Denis in 1859 (129) and by many later workers. A number of crystalline vegetable globulins, very slightly soluble in water but readily dissolved by salts, also became known during the latter half of the nineteenth century (112).

The solution of serum globulin by neutral salts was studied in detail by Hardy (130) and by Mellanby (131) in 1905. In the same year appeared the extensive studies of Osborne and Harris (132) on the solubility of the plant globulin, edestin, in salt solutions. All these investigators showed that the solvent action of salts on globulin increases with the valence of the salt ions. Mellanby formulated his results in a quantitative rule:

"Solution of globulin by a neutral salt is due to forces exerted by its free ions. Ions with equal valencies, whether positive or negative, are equally efficient, and the efficiencies of ions of different valencies are directly proportional to the squares of their valencies."

This is identical with the principle of the ionic strength, formulated in 1921 by Lewis and Randall.

Both Hardy and Mellanby observed that the solubility of serum globulin in a given solvent is not independent of the amount of saturating body but that the amount dissolved is nearly proportional to the total amount of globulin in the system. This observation indicates that the protein studied could not have been a pure chemical individual. Hardy, indeed, concluded from a variety of observations that globulin as such is not present in untreated serum, and suggested that "the balance of probability is . . . in favor of there being in serum some (possibly one) complex proteid which breaks down readily into fractions whose composition and properties depend upon the degree of dilution and the reagents used" (130, p. 327).

Because of the existence of such complex relationships, many investigators concluded that protein solutions in water, or protein crystals in apparent equilibrium with such solutions, could not be regarded as definite phases, in the sense of Willard Gibbs. The classical investigations of Sörensen (133) on egg albumin in ammonium sulphate solutions demonstrated, however, that this protein does essentially fulfill the requirements of the phase rule and gives a reproducible solubility, nearly independent of the amount of saturating body in the system, provided that pressure, temperature, salt concentration, and pH are all defined. More recently, Sörensen, largely as a result of extended studies on the serum proteins (134), gliadin (135), and casein (136, 137, 138), has modified his earlier view and has adopted a general conception closely related to that which Hardy had proposed for the special case of serum protein. According to this interpretation, "soluble proteins consist of a series of complexes or components, reversibly combined, which makes their constitution expressible by the ordinary formula A_xB_yC_z···A, B, C and so on each marking complete complexes, mainly polypeptides, yet in some cases also containing other groups, for example phosphorus groups, whereas the affixed indices x, y, z, and so on, mark the amount to which the indicated complex is present in the entire component system. Within each complex all of the atoms and atomic groups are linked together by main valencies, whereas the various complexes in the whole component system are comparatively loosely and reversibly knit together by means of the residual valencies which each component must be assumed to possess, and the strength and nature of which must depend on the chemical composition of the component in question as well as on its physical properties, above all on its dimensions and the resulting shape and surface. But all things considered, the linkage between the components must be supposed to be comparatively slight and of such a nature that alterations in the composition of the solution (salt content, hydrogen-ion activity, alcohol content, temperature) may give rise to reversible dissociations of the involved component systems and interchange of components between the same" (121, p. 18). Hence the solubility relations are complicated, and the amount dissolved should, in general, vary with the amount of saturating body.

The existence of such association and dissociation reactions for many proteins is borne out by other observations. Thus Burk and Greenberg (139) have shown from osmotic pressure measurements that hemoglobin in concentrated urea solutions dissociates into molecules of molecular weight of about 34,000, one-half the size of the molecule in water. Steinhardt (140), in studying the sedimentation velocity in the ultracentrifuge, and the diffusion of hemoglobin in water and urea solutions, has observed the same effect. The small molecules largely reassociate when the urea is removed by dialysis (unpublished observations). Ultracentrifugal studies on serum proteins (141) also reveal a remarkable series of association and dissociation reactions, depending on the concentration of the protein and salt present, pH, and other factors; and a series of other studies from Svedberg's laboratory (142) have shown somewhat similar effects for many proteins in markedly acid or alkaline solution.

Sörensen pointed out, however, that for egg albumin dissolved in aqueous salt solutions, not far from its isoelectric point "the dissociation tendency of this system is but slight" (121, p. 55). In other words, the conception of egg albumin as a single chemical individual, whose crystals form a definite phase, remains applicable as a good first approximation to the truth. The careful and searching study later made by Sörensen concerning horse carboxyhemoglobin (143) has led him to very similar conclusions concerning this protein also. A number of proteins studied in other laboratories

during recent years—such as human hemoglobin (144), myoglobin (145), fibrinogen (146), lactoglobulin (147), crystalline pepsin (148), and trypsin (149)—appear to belong to the same class of well-defined chemical individuals. All of these proteins are relatively small molecules; with the possible exception of fibrinogen, none appears to have a molecular weight above 70,000 under normal conditions. Further investigation should lead to the discovery of many more such proteins.

To obtain reproducible solubility measurements, such proteins must generally be studied fairly near their isoelectric points, preferably at low temperature, and in solvents having a minimal tendency to produce denaturation. If these conditions are carefully observed, the measurements obtained should form a dependable basis for the correlation of solubility with chemical structure.

9. The Solubility of Certain Proteins in the Absence of Salts

Very great differences in solubility in water are to be found among the proteins (Table XVI). Isoelectric egg albumin can remain in a clear solution at a concentration of 40 per cent in water; while globulins such as edestin and myosin have no easily measured solubility in pure water. Horse carboxyhemoglobin is only moderately soluble (17 gm./L) while human carboxyhemoglobin is considerably more soluble. The differences in water solubility among these proteins are thus as great as those to be found among the amino acids, from glycine to cystine.

Isoelectric egg albumin and hemoglobin can be equilibrated with 25 per cent ethanol without denaturation, provided the process is carried out at a low temperature (-5°) (Ferry, Cohn, and Newman (153)). The solubilities of these two proteins under these conditions are given in Table XVI. Egg albumin is at least three thousand times and hemoglobin approximately five hundred times as soluble in water at 25° as in 25 per cent ethanol at -5° . Part of this effect may be due to the difference in temperature (the temperature coefficient of the solubility of a protein in water, or its heat of solution, has apparently never been determined) but the change of medium is undoubtedly a far more important factor. These medium effects resemble those of alcohol in amino acid solutions, but are far greater. Even such a highly polar peptide as diglycine is less than four times as soluble in water at 25° as in 25 per cent ethanol at the same temperature (Table XI).

Table XVI

The Solubility of Certain Proteins in the Absence of Salt Near Their Isoelectric Points

Protein	Ref. Solubility in water gms./liter***		Approximate pH	Solubility in 25 per cent ethanol at -5° gms./liter Ref.		
Egg albumin		>400 >300	4.8 6.3	0.13 (153)		
CO-Hemoglobin, human. CO-Hemoglobin, horse.	(144) (151)	large 17	6.8 6.6	0.036 (154)		
Lactoglobulin** Pepsin†	(147)	0.3 to 0.9 0.3 to 0.5	5.2 (in 0.05 N HCl)			
Casein*	(114)	0.110	4.8			
Serum globulin*	(114)	0.076	5.4			
Zein*	(114)	0.054	5.7			
Edestin	(132)	very small	6 to 7			
Myosin*	(152)	very small	6 to 7			

^{*} These values were determined on preparations which probably did not consist of a single protein.

10. The Solubility of Proteins in Dilute Salt Solutions, Acids, and Alkalies

The studies of Hardy, Mellanby, and Osborne on globulins in dilute salt solutions revealed the principle of the ionic strength, and many general features of the solvent action of salt on globulins. Serum globulins, however, have no defined solubility, the amount dissolved varying with the amount of saturating body, and edestin is so insoluble in the absence of salt that the value S' (solubility at zero ionic strength) has never been exactly determined. Further and more exact studies required the use of a protein which should be a chemical individual, with a well-defined and reproducible solubility under constant conditions, both in water and in salt solutions.

In 1926, Cohn and Prentiss (155) showed that the oxyhemoglobin of the horse essentially fulfils these conditions. Its solubility

[†] This value for pepsin is from unpublished measurements of Dr. Jacinto Steinhardt. Values previously reported (148) are appreciably higher.

^{**} The precise value estimated for lactoglobulin depends on the method of extrapolation from measurements in dilute salt solutions. See footnote to Table XVII.

^{***} Data are for room temperature (18 to 25°).

in water (Table XVI) is higher than that of most globulins, but still relatively small, and neutral salts produce a well-marked and easily measured solvent action. Cohn and Prentiss showed the close analogy between the action of salts on oxyhemoglobin and their solvent action on certain inorganic electrolytes of high valence type and low solubility, which had been studied by Brönsted (156).

The study of the action of salts on oxy- and carboxyhemoglobin

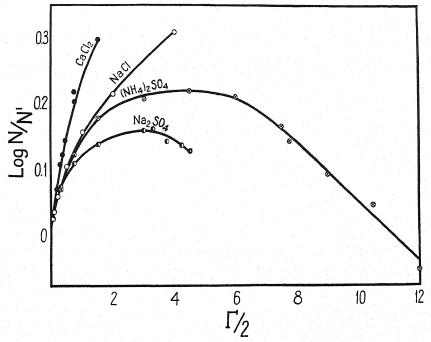


Fig. 16a. Solubility of cystine in salt solutions of varying ionic strengths. Data from T. L. McMeekin.

(Cohn, E. J., Chem. Rev., 19, 241 (1936).)

was greatly extended by Green (151, 157, 158) who determined, for a large variety of salts, both the solvent action at low salt concentrations, and the salting-out effect at high concentrations. The effect of pH and temperature was also studied. These studies represent probably the largest body of accurate experimental data yet available concerning the solubility of a single protein in a wide variety of salt solutions. Some of these data are represented in Fig. 16b. A similar figure showing the effects of salts on cystine is given in Fig. 16a. The qualitative similarity of the salt effects in

the two cases is immediately apparent, the solvent action of the salts decreasing in the order NaCl>(NH₄)₂SO₄>Na₂SO₄, and the form of the curves being generally similar. The magnitude of the effects on hemoglobin, however, is far greater than on cystine (note the difference in the scale of both abscissas and ordinates in Figs. 16a and 16b). Thus hemoglobin is approximately eleven times as

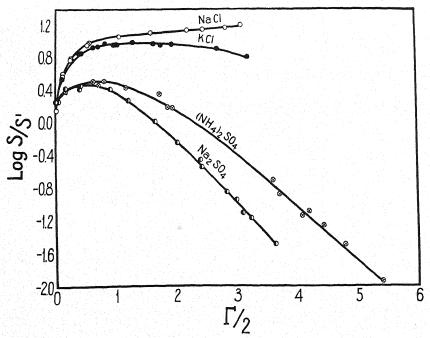


Fig. 16b. The solubility of hemoglobin in salt solutions of varying ionic strengths. Data from A. A. Green.

(Cohn, E. J., Chem. Rev., 19, 241 (1936).)

soluble in molar NaCl as in pure water, while cystine is less than half again as soluble in this medium as in water. The effect of salts on many other globulins is far greater than on hemoglobin. Thus Osborne and Harris (132) showed that edestin is approximately nineteen times as soluble in 0.8 M as in 0.4 M NaCl.

Theoretical interpretation of the effect of salts on the solubility of hemoglobin in water, following the treatment for dipolar ions outlined in Section II, is at present difficult. This is largely because of the great dielectric constant increment of hemoglobin (35,000 per mole or approximately 0.5 per gm.) and its large and changing solubility in various solvents. Through its effect on the dielectric

constant of the medium, hemoglobin will affect its own activity and that of the other substances present in the system, in a manner which is of undoubted importance but which cannot at present be calculated exactly. A protein too insoluble to affect the dielec-

Table XVII

The Solvent Effect of Very Dilute Sodium Chloride on Certain Amino Acids,

Peptides, and Proteins

(The solvent action of the salt is given by K_R '; other related molecular constants

are also tabulated)

Substance	$\begin{array}{c} \mathrm{Ref.} \\ \mathrm{for} \\ K_R{'} \end{array}$	Molec- ular weight	Molec- ular volume cc.	Number of dipole pairs	Dielectric constant increment, δ (Table IV)	$K_{R'} = \frac{\frac{D}{D_0} \log \frac{N}{N'}}{(D_0/D)C}$
Glycine Leucine Cystine	(107) (93) (93)	75.05 131.10 240.20	57.0 122.2 156.1	1 1 2	23 25	0.32 0.30 0.42
Diglycine Triglycine Lysylglutamic	(107) (107)	132.07 189.11	93.3 129.6	1 1	70 113	0.58 0.77
acid	(107)	275.20	211.5	2	345	1.16
Egg albumin Horse CO- hemoglobin	(153)	34,000 66,700	25,500 50,000	27 75	3,400	14 17
Lactoglobulin	(147)	39,000	29,000			38

Palmer, in studying lactoglobulin, followed earlier treatments, and plotted log S against the square root of C_{NaCl} , thus deducing a limiting solubility in water, S', of approximately 0.3 grams per liter. It has been found, however, that all the lower points on Palmer's curve are fitted (within ± 0.025 in log S) by the equation log $S=-0.03+38C_{\text{NaCl}}$. By this method, then, S' is calculated as approximately 0.93 grams per liter.

This table has already been given, in a slightly different form, by Cohn (93). The data for the amino acids and peptides are at 25° , those for egg albumin and hemoglobin are for -5° , and those for lactoglobulin are at 30° .

tric constant of the medium appreciably should give results more readily interpretable.

Several such proteins are known, one of which was recently isolated by Palmer (147) from milk. He showed that the fraction of milk protein which had been commonly called lactalbumin actually consisted largely of a readily crystallizable globulin, lactoglobulin, very insoluble in water (Table XVI) but readily dis-

solved by salt. Palmer showed that the solubility of the protein was essentially independent of the amount of saturating body. He measured its solubility in a series of dilute salt solutions, with results shown in Table XVII and in Fig. 17 (No. 7).

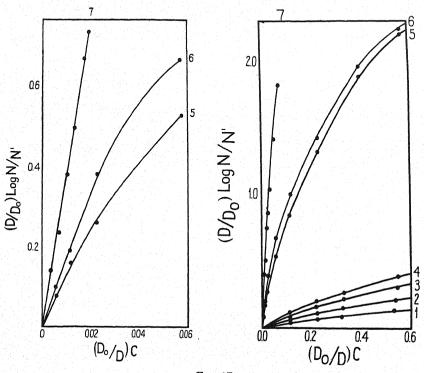


Fig. 17

(a) Solubility of proteins in very dilute salt solutions.

(b) Solubility of amino acids, peptides, and proteins in dilute salt solutions. The numbers attached to the curves indicate:

1. Glycine; 2. Diglycine; 3. Triglycine; 4. Lysylglutamic acid; 5. Egg Albumin; 6. Carboxyhemoglobin (horse); 7. Lactoglobulin.

(Cohn, E. J., Chem. Rev., 19, 241 (1936).)

Ferry, Cohn and Newman (153) studied the effect of salts on the solubility of egg albumin in 25 per cent ethanol at -5° . In the absence of salt, the egg albumin, under these conditions, is too insoluble to affect the dielectric constant of the medium appreciably (Table XVI), and small quantities of salt have a great solvent action. Under these circumstances, then, egg albumin behaves exactly as does a globulin in water. The solubility of carboxyhemoglobin in the same solvent at -5° is also very low (Table XVI) and

the solvent action of neutral salts is much greater than in water (154). The effect of salts on these two proteins in 25 per cent ethanol at -5° is shown, with Palmer's data on lactoglobulin in water, in Fig. 17 and Table XVII. The corresponding data for some amino acids and peptides (compare Fig. 13) are also given.

The solvent action of very dilute salts on these proteins is directly proportional to the ionic strength (Fig. 17a) as would be expected from theory (Equations 30 to 34 incl.). Furthermore, the limiting slope, $K_{R'}$, of the solubility curves is far greater for the proteins than for the amino acids and peptides, being more than one hundred times as great for lactoglobulin ($K_{R'}=38$) as for glycine ($K_{R'}=0.32$). On the other hand, the value for egg albumin ($K_{R'}=14$) is only about twelve times as great as for the very polar peptide, lysylglutamic acid; so that the synthesis of somewhat larger peptides of the same type may reveal behavior closely approximating that of some proteins in this respect. But the results at present available appear to indicate clearly the underlying similarity in the action of salts on amino acids, peptides, and proteins.

The high values of K_R' for the proteins in Table XVII are undoubtedly closely connected with their high dielectric constant increments and dipole moments; but their exact relation to the electrical configuration of the protein molecules remains to be determined. For all of these proteins, the value of the salting-out constant, K_s , must be considerable; hence the observed slope, K_{R}' , must be less than the ideal slope, K_{R} , of the curve determined by Coulomb forces alone (Equation 34). Furthermore, K_R is determined according to Kirkwood's theory (41), not only by the net dipole moment of the molecule, but by more complicated distributions of electric charge, whose resultant effects are described in terms of multipole moments (quadrupole, octupole, and so forth). For a molecule like glycine which carries a dipole made up of a single positive and a single negative charge, the center of the dipole coinciding nearly with the center of the molecule, all multipole moments above the dipole will be practically zero, and the simplified equation (30) is applicable. Proteins, however, carry a great number of charges which probably are widely distributed over the surface of the molecule, and the center of the resultant dipole (obtained by vector summation of all the individual positive and negative charges) will not always coincide with the center of the molecule. Kirkwood's treatment, however, yields equations applicable in principle for the calculation of K_R in systems containing

any arbitrary number and distribution of charges in a spherical molecule. The problem remains complex; but studies of the solvent action of salts on proteins, considered in connection with titration curves, analysis of amino acid content, dielectric constant determinations, X-ray diffraction studies, and other lines of evidence, should lead to a far deeper understanding of the electrical configuration of the protein molecule.

Since neutral salts dissolve proteins, the converse effect should also be expected. This was shown experimentally by Pauli and Stenzinger (159) who found that serum albumin, pseudoglobulin. and hemoglobin definitely increased the solubility of calcium sulfate in water, the solvent action of these proteins increasing in the order named. Stone and Failey (160) studied the effect of several proteins on the solubility of thallous chloride. Isoelectric egg albumin and hemoglobin exerted a slight solvent action on this salt, the increase in log S_{TICI} being proportional to the protein concentration, as would be theoretically expected. Acid and alkaline salts of hemoglobin, egg albumin, edestin, and excelsin showed a much larger solvent action than the isoelectric proteins, the effect being proportional in these cases to the square root of the concentration of protein salt. Such a relation is also to be expected theoretically, since the protein carries a net charge except at its isoelectric point. Both the theory of Debye and Hückel (95) and the extension of the theory by Kirkwood (41) indicate that the square root law should apply to such a system.

(4) Solubility of Proteins in Acids and Alkalies. The solubility of most slightly soluble proteins, like that of amino acids (161, 162), is minimal at or near their isoelectric points, and (in the absence of salt) addition of either acid or alkali generally causes an increase in solubility. This was shown by Osborne (112) for many vegetable proteins and by Hardy (130) for serum globulin. For proteins as for amino acids, the dipolar ion, carrying zero net charge, is generally less soluble in the absence of salt than the positive or negative ions produced from it by the addition of acid or alkali. Change in solubility with pH is thus associated with change in the net charge on the protein molecule; that is, with the relative steepness of the titration curve in the given region.

Cohn and Hendry (163) found that the solubility of casein in the presence of small amounts of alkali could be described by a simple equation, and Green (158) has given this equation in a more general form, which describes satisfactorily the behavior of both casein and hemoglobin on both sides of their isoelectric points. In this treatment it is assumed that at any pH throughout the range considered, one form of the protein in solution (presumably a form with zero net charge) is directly in equilibrium with the saturating body. The concentration of this form may be taken as approximately independent of pH in all the systems considered, and is denoted by S_n .²⁴

Then the total solubility of the protein as a function of pH may be described by the equation:

$$\frac{S}{S_n} = \frac{H^2}{K_1 K_2} + 1 + \frac{K_3 K_4}{H^2} \tag{35}$$

where H is the hydrogen ion activity (the negative antilogarithm of the measured pH) and the K's are constants at any given ionic strength.²⁵ In solutions distinctly alkaline to the isoelectric point, the first term on the right of equation 35 may be neglected, and

$$\frac{S}{S_n} = 1 + \frac{K_3 K_4}{H^2} \tag{35a}$$

In solutions distinctly acid to the isoelectric point, the last term may be neglected and

$$\frac{S}{S_n} = \frac{H^2}{K_1 K_2} + 1 \tag{35b}$$

 24 S_n is not identical with S', the total solubility of the isoelectric protein in the absence of salt, since even at the isoelectric point some protein anions and cations (the sum of the number of positive and negative charges being of course equal) will be present, as well as the dipolar ion of zero net charge. Thus for horse carboxyhemoglobin, Green has estimated S' as 17, and S_n at zero ionic strength as 11 grams per liter.

²⁵ The theoretical significance of equation (35) is still obscure. It is closely related in form to the equation describing the solubility of a diaminodicarboxylic acid like cystine as a function of pH (Sano (162)), which may be written:

$$\frac{S}{S_n} = \frac{H^2}{K_1 K_2} + \frac{H}{K_2} + 1 + \frac{K_3}{H} + \frac{K_3 K_4}{H^2}$$
 (36)

This equation describes very satisfactorily the solubility of cystine in acid and alkali, and may readily be derived, as a good approximation, from simple theoretical considerations (see Chapter XI). Equation (35) is similar in form, but lacks the terms in H and 1/H. Why such a simplified equation (or indeed any simple equation) should describe satisfactorily the solubility of a protein as a function of pH is not yet obvious. For the present, equation (35) must be regarded simply as a very useful empirical tool for describing the facts.

Equation (35) proves applicable to case and to hemoglobin in the presence of salt also, provided that S_n and the K's are regarded, not as constants, but as functions of the ionic strength. It is found for hemoglobin that S_n at first increases on addition of salt, then passes through a maximum and, in concentrated solutions, decreases (the salting-out effect). This corresponds to the characteristic solubility curve for a globulin in solutions of varying ionic strength. The coefficients K_1K_2 and K_3K_4 increase on addition of salt, slightly in hemoglobin solutions, very markedly in case solutions. This increase of the constants with salt concentration has a twofold consequence: (a) the value of H for minimum solubility is given, for a system in which equation (35) holds, by the relation:

$$H^4 = K_1 K_2 K_3 K_4 \tag{37}^{26}$$

Since all of the K's increase with increasing ionic strength, the value of H at minimum solubility must increase also; or the pH of minimum solubility decreases. This effect is comparatively slight for hemoglobin; but for casein, Green has calculated from the data of Sörensen and of Linderström-Lang that the pH of minimum solubility shifts from 4.8 in the absence of salt to 4.06 at ionic strength 0.1.27 (b) Solubility, at constant pH, on the alkaline side of the isoelectric point is markedly increased by salt at low or moderate salt concentrations (since both Sn and K_3K_4 in equation (35a) increase with ionic strength). This effect is found in both casein and hemoglobin. On the acid side of the isoelectric point, S_n and K_1K_2 both increase with increasing ionic strength (Equation (35b)), the former factor tending to increase the solubility S. the latter to decrease it. In hemoglobin, at constant pH, on the acid side of the isoelectric point, the resultant effect is a very slight increase in solubility with increase in salt concentration, followed by salting-out which begins at a relatively low salt concentration. In casein the increase of K_1K_2 is very rapid, and the casein cation

²⁶ At the point where S is a minimum, dS/dH must be zero, and from (35):

$$\frac{dS}{dH} = S_n \left(\frac{2H}{K_1 K_2} - \frac{2K_3 K_4}{H^3} \right) = 0 \tag{38}$$

from which equation (37) follows. It is clear from the nature of equation (35) that equation (38) gives the conditions for a minimum and not for a maximum.

²⁷ Whether the isoelectric point as determined from cataphoresis measurements shifts similarly is a problem not yet definitely settled. The whole question is closely related to the distinction which has been drawn between the "isoelectric" and the "isoionic" points of a protein. See Sörensen, Linderström-Lang, and Lund (164) and Adair (165).

is salted out very rapidly even by the smallest concentrations of salt, in contrast to the anion which is readily dissolved by salt under the same conditions.

Phenomena of this type had been clearly perceived by earlier observers of other proteins. Thus Osborne (112) wrote that "edestin dissolved in the least possible quantity of hydrochloric acid necessary for its solution is precipitated by traces of most mineral salts, but a slight excess of acid requires the addition of more salt for precipitation. The precipitation of most seed proteins by acids ... depends largely on the presence of mineral salts in their solutions." Similarly Hardy (130) showed clearly that the addition of salt to serum globulin shifts the point of minimum solubility to a more acid range, the magnitude of the shift depending on the salt employed, as well as on its concentration. In summarizing the combined effects of salts, acids, and alkalies on serum globulin, he concluded, "One feature of fundamental importance, which is never obscured . . . is the antagonism between the solvent actions of salts and acids, and the additive nature of the combined solvent action of salts and alkali." This statement exactly characterizes the behavior of many other proteins in acid and alkaline salt solutions. Thus myosin (152, and unpublished observations), in solutions alkaline to pH 6.0 is dissolved by both salts and alkalies, the solvent actions of the two tending to reinforce one another; from about pH 4.7 to 6.0, myosin is virtually insoluble at any salt concentration; at reactions acid to pH 4.6 it forms a clear solution, which, however, is precipitated by even small additions of neutral salts.

Thus the "antagonism between the solvent action of salts and acids" on proteins, long ago pointed out by Osborne and by Hardy, appears to be a wide-spread phenomenon, found in proteins in very various types. It may be due to the formation of insoluble acid salts of these proteins, as Osborne suggested for the vegetable globulins;²⁸ but this interpretation cannot be regarded as established until the chemical composition of the precipitated protein phase in such systems has been accurately determined. However

²⁸ Casein in the presence of small amounts of calcium hydroxide forms an insoluble calcium salt which dissolves on the addition of further calcium hydroxide and forms a more soluble alkaline salt (166, 114, 167). Undoubtedly many proteins form similar insoluble salts with alkalies, but this phenomenon seems to be rather less common than the formation of insoluble protein complexes in the presence of salts and acids.

the phenomenon may be interpreted, it gives rise to a "salting-out" of certain protein cations even in very dilute salt solutions. In concentrated salt solutions, salting-out is a much more general phenomenon; protein cations, anions, and dipolar ions are all precipitated by sufficiently high concentrations of suitable salts. To this phenomenon we may now turn our attention.

11. The Solubility of Proteins in Concentrated Salt Solutions; the Salting-out Effect

Except under the special conditions just described, the solubility of albumins and globulins is seldom reduced by salts except in concentrated salt solution. In this respect, their behavior is in marked contrast to that of most uncharged organic molecules, and of such isoelectric amino acids as leucine and tyrosine, whose solubility is diminished by even small additions of salt. It is remarkable that the salting-out of proteins in concentrated salt solution can nevertheless be described by an equation of the same form as that describing the solubility of many other molecules in dilute salt solution. If the solubility, S, of the protein is expressed in grams per liter, that equation may be written²⁹

$$\log S = \beta' - K_s' \frac{\Gamma}{2} \tag{38}$$

 $K_{s'}$, the salting-out constant, gives the slope of the curve while β' is the extrapolated intercept of the linear portion of the curve on the ordinate axis. Actually, of course, this equation describes the data only over a limited range where the solubility of protein is sufficiently small. Thus in Figure 16b the curves for $\log S$ (carboxyhemoglobin) in NaCl and KCl do not show any significant decrease of solubility even in a highly concentrated salt solution. Even the curves for $(NH_4)_2SO_4$ and Na_2SO_4 begin to descend linearly only at fairly high values of $\Gamma/2$.

²⁹ If the solubility is expressed as grams of protein per thousands grams of water, a similar logarithmic equation also applies.

$$\log S = \beta - K_s \mu \tag{38a}$$

or, if solubility is expressed as mole fraction of protein,

$$\log N = \beta - K_s \Gamma / 2 \tag{38b}$$

The fit of the equations to the data is almost equally good in all these expressions, but the values of the constants involved (β or β' , and K_s or K_s') will naturally be somewhat different according to the form of expression which is used.

 β' is the logarithm of a purely hypothetical protein solubility obtained by backward extrapolation of the linear portion of such curves. Equation (38) was first pointed out by Cohn (114) in 1925, who showed that it fitted closely the data of Chick and Martin (168) and of Sörensen (133) on the solubility of egg albumin in ammonium sulfate, and Sörensen's data (134) on pseudoglobulin

Table XVIII

Values of the Salting-Out Constant, Ks', for Amino Acids and Proteins

		Salt					
Substance	Ref.	NaCl	(NH ₄) ₂ - SO ₄	Na ₂ - SO ₄	K ₂ HPO ₄ and KH ₂ PO ₄	Mg- SO ₄	Na ₃ Citrate
Cystine*			0.05				
α -Aminobutyric acid.	(108)	0.04					
Leucine	(101)	0.09					
Tyrosine	(104)	0.31					
Lactoglobulin	(147)			0.63			
Hemoglobin, (horse).	(157)		0.71	0.76	1.00	0.33	0.69
Hemoglobin, (man)	(144)				2.00		
Myoglobin	(145)		0.94				
Egg albumin	(133, 114)		1.22				
Fibrinogen	(146)	1.07	1.46		2.16		

Some of these values are slightly revised from earlier estimates (114, 37), where certain data have been rechecked and recalculated.

in the same solvent. The same relation has since been shown to hold for a considerable number of well-defined proteins.

While β' is markedly influenced by pH and temperature, the salting-out constant K_s' , for a given protein and a given salt, is independent of both these factors within the experimental error. Values of K_s' may thus be employed to characterize the interaction of a particular protein with a concentrated solution of a given salt. A number of such K_s' values are given in Table XVIII; a few values for amino acids are also given for comparison.

In general the values of K_s' for the proteins in Table XVIII are ten to fifty times as large as for the amino acids (with the exception of tyrosine, whose salting-out constant is remarkably large). In a logarithmic equation, differences of this magnitude in K_s' produce very great differences in the effect of salts on solubility. Thus the solubility of leucine is reduced approximately 11 per cent by 0.5 N

^{*} From the data shown in Figure 16a.

sodium chloride, while the solubility of fibrinogen is reduced by nearly 1000 per cent by a change in the ionic strength of a phosphate buffer solution from 1.5 to 2.0. Values of K'_s for different proteins and the same salt, or for different salts and the same protein, have not been found to differ by a factor more than about two. Thus the value of K_s' for fibrinogen is slightly more than twice as great as for horse hemoglobin, whether potassium phos-

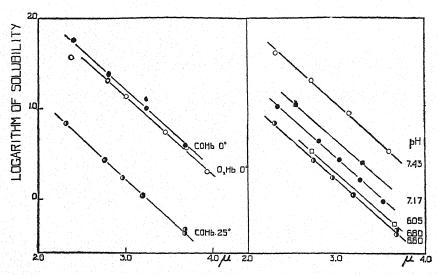


Fig. 18. The solubility of hemoglobin in concentrated phosphate buffers of varying temperature and pH.

(Green, A. A., J. Biol. Chem., 93, 507 (1931).)

phate buffers or ammonium sulfate be used as the precipitating agent; and the efficiency of potassium phosphate as a precipitating agent for fibrinogen (as measured by the value of K_s) is almost exactly twice as great as that of sodium chloride.

For the proteins as for simpler molecules, salts containing multivalent anions—phosphates, sulfates, and citrates³⁰—are the most efficient salting-out agents. Sodium sulfate is definitely more effective than ammonium sulfate, but the higher solubility of the latter salt often renders its use more convenient. Sodium and potassium chlorides salt out fibrinogen and also myosin (152), although less effectively than sulfates; but their salting-out effect on hemoglobins and albumins, even if present, does not lead to a marked de-

³⁰ The normal carbonates, which in general have a powerful salting-out action, cannot well be employed with proteins on account of their high alkalinity.

crease in protein solubility, even in their saturated solutions. The salting-out tendency of calcium chloride, and presumably of other salts containing bivalent cations, is apparently still smaller.

In contrast to the constancy of K_s ' for a given protein and a given salt, the variation of β with pH and temperature is very marked. The variation with pH reflects the influence of the ionization of the protein upon solubility in concentrated salt solutions. Plots of

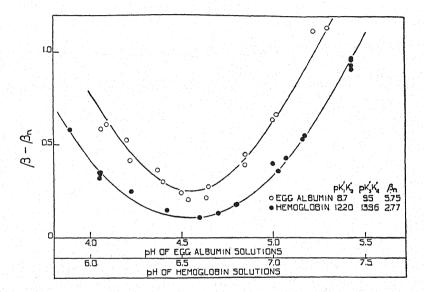


Fig. 19. The solubility of hemoglobin and of egg albumin in concentrated salt solutions of varying pH.

The ordinate shows the change in the logarithm of protein solubility as a function of pH.

Data on egg albumin in ammonium sulfate solutions from Sörensen and Höyrup (133).

Data on hemoglobin (horse) in phosphate buffers from Green (157, 158). (Green, A. A., J. Biol. Chem., 93, 524 (1931).)

log S against ionic strength at various pH's yield a series of parallel lines, as shown in Fig. 18, taken from the work of Green (157) on hemoglobin. At a given ionic strength, solubility as a function of pH may be described by equation (35) for both hemoglobin and egg albumin. Figure 19 shows the variation of β with pH in concentrated salt solutions for these two proteins. The curves passing through the experimental points are calculated from equation (35), using suitable values of S_n and of the constants K_1K_2 and K_3K_4 . Since equation (38) holds for these systems, and K_s' is independent

of pH, these curves also represent the variation of the logarithm of the actual protein solubility (log S) at constant ionic strength, as a function of pH.³¹

Sörensen (143) has shown that the solubility of horse carboxy-hemoglobin in concentrated ammonium sulfate passes through two minima, one near the isoelectric point (pH 6.6), the other in acid solutions at pH 5.4. He interprets this second minimum as due to the formation of an insoluble hemoglobin sulfate, containing twelve or thirteen sulfate ions per mole of hemoglobin; an interpretation recalling Osborne's views concerning the precipitation of the vegetable globulins by the combined action of salts and acids.

The value of β' is very sensitive to temperature, as well as to pH. A series of log S-ionic strength curves at various temperatures in the salting-out range forms a series of parallel straight lines (Fig. 18). Horse carboxyhemoglobin is approximately ten times as soluble at 0° as at 25° under these conditions, although at low salt concentrations its solubility is increased by rise of temperature. In myosin (152) the decrease in solubility with rise of temperature. in the salting-out range, is even more marked; a solution which is highly soluble at 0° may be almost completely precipitated at 25°. The effect of temperature on the solubility of egg albumin, on the other hand, is relatively slight; the measurements of Sörensen (133), discussed by Green (158), indicate that solubility passes through a slight minimum at about 20°. Vegetable seed globulins. after extraction, have frequently been crystallized (169) from salt solutions of moderate concentration, simply by cooling the warm saline extract. These proteins, then, are much less soluble under the given condition at low than at high temperature.

The varying response to pH and temperature of different proteins may prove of practical importance in their separation. Constituents of a complex protein mixture which are very similar in solubility at a given pH and temperature may be affected very differently by the variation of one or the other of these factors. A vast number of investigations of the fractionation of the serum proteins by salting-out have been made; of those in relatively re-

 $\frac{S}{S_n} = \frac{\text{antilog } \beta}{\text{antilog } \beta_n}.$

The method of calculating β_n is explained in detail by Green (158). Its absolute value is immaterial in the present connection, since it does not affect in any way the *change* in log S with pH, shown in Figure 19.

³¹ The ordinate in Figure 19 is not β , but β minus a constant, β_n . This is defined by the relation:

cent years the work of Howe (170) may particularly be mentioned. Still more recently, Butler and his collaborators (171, 172) (see also Kydd (173)) have analyzed the salting-out of serum proteins in phosphate solutions with the aid of equation (38), and have differentiated several distinct steps in the process, presumably corresponding to the separation of different protein fractions. Recent studies of serum with the aid of the ultracentrifuge (141) indicate, however,—as Hardy long ago suggested—that the effect of salts on serum proteins is very complex, so that no final interpretation of the phenomena can yet be offered.³²

The underlying mechanism of the salting-out effect is undoubtedly complex. Certainly however, Hofmeister (127) emphasized an important element of the truth in suggesting that the phenomenon was due to a "dehydration" of the protein by the added salt. This conception, couched in more explicit and quantitative terms, forms the basis of Debye's theory of salting-out (98, 100), according to which the salt ions attract around themselves the more polarizable molecules of the medium (in this case water), thereby squeezing out other components such as proteins. The specific influences of different salts, as enunciated by Hofmeister in his studies on proteins, are found also in the action of salts on amino acids, and indeed on the simplest gases (99, 174).

The striking qualitative similarity in the action of salts on the solubility of cystine and of hemoglobin (Figs. 16a and 16b) may again be recalled in this connection. The salting-out effect in the action of salts on amino acids is apparent (see equation (34)) even in very dilute salt solutions; in proteins, likewise, its significance probably will prove to be even greater than earlier investigations would have led us to suspect. The elucidation of the nature of the effect is not a special problem, confronting the colloid chemist only; it is rather a problem of extraordinarily wide scope in the general theory of solutions.

²² The reader will find a valuable discussion of the solubility of proteins in the monograph by Pauli and Valkó (175). In this connection see also McBain, J. W., and Jameson, E., *Trans. Faraday Soc.*, **26**, 768 (1930).

Recently J. Steinhardt of this laboratory (see *Proc. Amer. Soc. Biol. Chem.*, CXV (1938)) has shown in very careful studies that "the isoelectric solubility of crystalline pepsin, as of many other proteins, is a function of the ratio of saturating body to solvent volume." He has derived an equation which describes the data very accurately, and shows that the results can be explained on the assumption "that protein crystals are solid solutions in which the protein mole fraction is low, as a consequence of high molecular weight."

For another important new study on protein solubility see (176).

I am indebted to Professor Edwin J. Cohn for advice and criticism which has been a constant aid to me in the preparation of this chapter. I have made extensive use of Dr. Cohn's recent article in Chemical Reviews. Also I owe much to valuable discussions with Professor Jeffries Wyman, Jr., Dr. T. L. McMeekin, Dr. Jesse P. Greenstein, and Professor Ronald M. Ferry.

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CHAPTER XVII

RELATION OF PROTEINS TO IMMUNITY

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1. INTRODUCTION

In the interactions between infectious agent and host and in the complex physiological processes resulting in resistance or immuity to disease the proteins are of the utmost importance. With the carbohydrates and lipids they form the chief barriers of defense of the animal body and make up the living protoplasm of the invading microorganism.

Instead of an invading microorganism, let us start with a simpler example. If one should add a drop of dilute egg albumin solution to the serum of a normal rabbit there would be no change, no evidence of any interaction. But if this foreign protein, egg albumin, be injected into the same rabbit an alteration slowly takes place in the reactivity of the animal. The foreign protein may be termed an antigen since it sets up changes in the rabbit resulting in the appearance in the animal's serum of new substances. These appear to be modified serum globulins and are termed antibodies. They are capable of reacting chemically with the antigen if this is introduced again, and removing it from the blood stream. After antibodies are formed, a new sample of the rabbit's serum, treated as before with a drop of egg albumin solution, becomes turbid and a precipitate composed of antigen and antibody settles out. The reaction, in vivo as well as in vitro, is a specific one in that the antibody reacts only with egg albumin and not with other proteins such as horse serum albumin. Similarly, horse serum albumin when injected into a rabbit gives rise to antibodies which are specific for the antigen used and do not react, for instance, with egg albumin or any other egg protein.

2. THE PRECIPITIN REACTION

The reaction which has just been described is called the *precipitin reaction*, since the union of antigen and antibody is made

evident by the formation of a precipitate. If the antigen is in particulate form, such as the collection of antigens contained in a suspension of bacteria, combination with antibody is made evident by a clumping, or agglutination, of the bacteria. The precipitin reaction, as well as other indirect or even in vivo evidences of antigenantibody combination which will be discussed later in the chapter, may be employed for the differentiation of proteins or for the identification of a protein of which the source is unknown or the properties are such that an immunological test is more convenient than a more purely chemical identification. The description in the preceding paragraph should serve to indicate roughly the procedure used.

While the specificity of the precipitin reaction is such that proteins which are only distantly related to the antigen used in producing the antiserum do not react, corresponding proteins of closely related species, such as crystalline duck egg albumin and crystalline hen egg albumin often cross-react in antisera to either protein. Even in such a case the proteins may usually be distinguished from each other, for addition of small amounts of the cross reacting protein to the antiserum produced with the aid of the other usually results in a point being reached at which the cross reacting protein no longer precipitates the antiserum, while the homologous protein still reacts with the portion of antibody which is left.

In the present chapter it is proposed first to recount some of the chemical studies which have led to a clearer understanding of the function of proteins as *antigens*, then to develop similarly the present conception of *antibody*, and finally to discuss such chemical knowledge of antigen-antibody interactions as is available.

3. THE ANTIGEN

Until recently it was considered that only intact protein or very slightly degraded protein could function fully as an antigen when introduced into animals and directly stimulate the production of antibodies (1). There is now evidence, however, that specific poly-saccharides and perhaps even certain lipids may act as antigens. With these exceptions it is evident that most antigens are proteins of varying degrees of complexity. It may likewise be said that most proteins are antigens, the common exception being the degradation product, gelatin. This is possibly due to its low content of the aromatic amino acids. Zein, which lacks tryptophane, lysine, and glycine but contains tyrosine and phenylalanine, and casein, which

lacks cystine and glycine but contains the aromatic amino acids, (see Table IV, Chapter IV) are antigenic. Heat-coagulated proteins are also said to be non-antigenic, but a denatured protein may function as an antigen if it can be brought into solution.

Landsteiner, who has developed much of the present-day knowledge of the chemical basis of specificity, has introduced the useful term hapten for that portion of a complex antigen which determines the serological specificity (2). A hapten fragment of an antigen, as the determinant of specificity, is capable of reacting with antibodies produced by injection of the whole antigen, but by itself rarely stimulates the production of antibody. With the aid of synthetically conjugated azoproteins it was shown that the diazotized aromatic amine is the hapten which determines the specificity of the resulting compound antigen, particularly if it contains an acid group; for regardless of the source of the protein the specificity of the new azoprotein is the same. Thus arsanilic acid (see Fig. 1c) or its sodium salt, atoxyl, diazotized and coupled with horse serum forms atoxylazo horse serum. This, injected into rabbits, gives rise

$$\begin{array}{c|c} NH_2 & & N:N & \\ \hline \\ AsO_3H_2 & & AsO_3H_2 & \\ c & & b \\ \hline \\ HO_3S & OH & & OH \\ \end{array}$$

Fig. 1

(Heidelberger, M., *Medicine*, 12, 279, 1933; Harvey Lectures, 28, 184, 1933; Text Book of Biochemistry, by Harrow and Sherwin, Philadelphia, 1935, p. 389.)

to antibodies which react equally well (as far as shown by qualitative tests) with the homologous antigen or with atoxylazo chicken serum, although native horse serum and chicken serum are each species specific and the antisera to them do not cross-react. Since the hapten introduced enters and modifies the tyrosine groupings (as in Fig. 1 d) and perhaps other cyclic groups of the protein, it would appear that these amino acids—chiefly tyrosine and histidine—play a dominant rôle in the specificity of antigens, and when

altered by the introduction of other substances, permit the establishment of a new specificity characteristic of the entering group. or hapten. Alteration of other acidic or basic groups by acylation. methylation, or addition of phenylisocyanate also changes the specificity, so that tyrosine and histidine are not the only groups concerned with the specificity of proteins (3). Denaturation also results in a partial change in specificity. The arrangement of the component amino acids must be of consequence, and in this connection, also, the recent work of Astbury, Bergmann, Waldschmidt-Leitz, Wrinch (see Chapters VI and VII) on the arrangement and number of the amino acids in proteins is of the utmost importance. Evidence has been presented that the "field of force" surrounding a molecular grouping is responsible for its specificity as a hapten, since groupings with similar fields of force are difficult to differentiate immunologically (4). However, there is also much against this view (5).

A class of haptens which is of great importance in the study of immunity is that of the specific polysaccharides, a distinctive group of carbohydrates which are capable not only of combining with antibodies but of precipitating them as well (6). Specific polysaccharides are mentioned here since they appear to exist in the bacterial cell at least in part in combination with protein, forming, in the case of encapsulated microorganisms such as *Pneumococcus*, the dominant, type-specific antigen of the cell. It would seem that nearly every species of bacterium is possessed of one or more specifically reactive polysaccharides, and although the connection of these carbohydrates with microbic structure, virulence, and immunological reactions is not always as clear as in the case of *Pneumococcus* (7), they play a definite and often determining rôle in bacterial specificity.

The protein antigens which are directly involved in infectious disease and the manifestations of immunity to such disease are still among the least known and most poorly characterized. Many pathogenic microorganisms not only build up proteins as an integral part of their cell protoplasm, but also secrete into the culture medium highly poisonous products, or toxins. Toxins are strongly antigenic, and the antibodies to which they give rise, the antitoxins, are of consistent therapeutic efficacy. Owing to the paramount importance of toxins in many diseases—diphtheria and tetanus are only two examples—numerous attempts have been made to isolate them, but these have succeeded only recently. As a result

of three independent investigations diphtheria toxin (8) appears to be an easily denatured protein of low molecular weight. Its isoelectric point is at about pH 4.1, and about 0.00045 mg. corresponds to one flocculation unit with antibody (L_f) . $[\alpha]_D$ lies between -40° and -50° . About 14 per cent of the total nitrogen reacts as amino nitrogen, and about one-third of this disappears in the reaction with formaldehyde to yield anatoxin. Correlation of the toxicity with chemical constitution has not yet been possible.

Also vaguely characterized are the proteins of the bacterial cell. The classical method for their preparation involved extraction of bacteria with weak alkali and precipitation of the extracted protein with acetic acid. It was recognized that the product obtained in this way showed some of the properties of nucleoproteins and of mucoproteins. Recently, however, it has been possible to make a tentative and imperfect separation of the complex mixture of antigens in the bacterial cell (9), carrying out all operations in the cold in order to minimize the action of the active enzymes known to be present. By means of a preliminary acidification the mixture of protein salts in the cell is brought to the isoelectric point, or below. An extraction with buffer at pH 6.5 then removes the most strongly acid fraction or fractions (D) (see Table I) of the cell proteins, while subsequent successive extractions with increasingly alkaline solutions remove protein fractions (E, F, G, K, Table I) of lower acidity. In this way a number of fractions have been obtained

Table I
Properties of Bacterial Protein Fractions

Fraction	$[\alpha]_D$	N	P	Basic ash	
	degrees	per cent	per cent	per cent	
. Streptococcus H	emolyticus, C 20	3			
23D	-10	15.8	3.2	0.4	
E	-13	14.8	2.7	1.5	
18F	-37	13.9	1.4	0.8	
G	-51	14.1	0.7	0.3	
ĸ	-57	13.9	0.1	1.0	
2. Tubercle Bacill	us, Human Strai	in, H ₃₇			
704D	-57	14.5	5.0	2.4	
E	-39	16.1	2.2	0.3	
F	-46	15.9	1.4	0.3	
G	-46	15.4	1.5	0.5	
K	-53	15.8	0.6	0.3	

from the hemolytic streptococcus and the tubercle bacillus. and the products have been further fractionated by partial precipitation with sodium or ammonium sulfate. In each group several of the fractions showed different immunological and chemical properties from those of other fractions. For example, the neutral-extractible fraction (D) from a scarlatinal strain of Streptococcus hemolyticus proved to be a dextrorotatory or weakly levorotatory nucleoprotein (or proteins) containing 3 to 4 per cent of phosphorus. It is antigenically distinct from the levorotatory fractions extracted by alkaline solutions. It is also extremely labile, splitting off a portion of its nucleic acid even in N/200 sodium hydroxide solution. It was concluded that at least the greater part of the nucleic acid in this fraction was present in an ester-like linkage. On alkaline hydrolysis the protein portion resembles the more alkaline-extractible fractions so closely as to be inseparable from these if the initial extraction is made with weak alkali as in the classical method.

After removal of the fraction soluble in neutral buffer the protein fractions extracted by successive solutions of increasing alkalinity contain less and less phosphorus, and in general are increasingly levorotatory as the phosphorus decreases. It was not possible to detect definite antigenic differences between these fractions.

In general, the streptococcus protein fractions contained more carbohydrate than that accounted for as nucleic acid (10), and, when injected into animals, gave rise to antisera containing anticarbohydrate as well as anti-protein. For recent streptococcus antigen fractionations see (10a).

Protein fractions prepared similarly from rapidly defatted tubercle bacillus cells showed even more marked immunological differences than did the streptococcus derivatives. In the tubercle bacillus fractions, also, a larger proportion of the nucleic acid present appeared to be uncombined. Corresponding protein fractions of the human and bovine strains of tubercle bacillus seem to be almost identical, but those of the avian and timothy grass strains are more easily distinguished from each other and from the human strain fractions by their serological, if not their chemical, reactions.

The liquid medium upon which tubercle bacilli have grown for some time is called *tuberculin*, and contains a complex mixture of proteins and carbohydrates derived from the bacilli. The great sensitivity of tuberculosis patients and those who have been in con-

tact with the disease to tuberculin has been referred to the protein constituents, and these have been studied in some detail (11). Their composition is given in Table II, together with that of water soluble and alkali soluble proteins of the tubercle bacillus cell (12).

Table II

Analysis of Tubercle Bacillus Proteins

	"Water soluble"	"Alkali soluble"	Tuberculin, TPT	
	per cent	per cent	per cent	
N	12.6	13.8	14.6	
P_2O_5		0.9		
S		0.65		
Cystine		0.9		
Tryptophane	1.5	1.9		
Tyrosine		0.9		
Arginine	8.7	7.6		
Lysine	3.1	5.5		
Histidine	4.7	2.4		
Total basic N (% of total				
N)			14.4	
Arginine N (% of total				
N)			8.4	

The agent specifically causing transmissible lysis of certain bacteria, or "bacteriophage" appears to be definitely antigenic. Methods for the purification of bacteriophage have been devised (13) and these have led to the isolation of nucleic acid-containing proteins which appear to be the actual 'phage. The particle size of staphylococcus 'phage varied greatly with the concentration. A possible autocatalytic mechanism for the formation of 'phage and virus is discussed in (13c).

Evidence is also accumulating that certain members of the plant virus group, at least, are not living agents but proteins. The causative agent of tobacco mosaic disease appears to be a crystalline protein which also contains nucleic acid (14). The molecular size is extraordinarily high, about 15,000,000–20,000,000, and as a result it has been found possible to separate this and other viruses by combinations of high-speed and low-speed centrifugation.

4. THE ANTIBODY

Although writers on immunology are still to be found who prefer to consider antibodies as ideas, rather than chemical substances, there is now much evidence that antibodies actually are modified serum globulin. Occasional claims are still put forward that protein-free antibodies have been obtained but their analysis indicates that the oft-repeated demonstration has been forgotten that chemical tests for protein fail at dilutions at which biological reactions such as anaphylaxis and bacterial agglutination (see below) readily occur. Until such claims are accompanied by the isolation of weighable amounts of protein-free antibody they can carry little conviction.

On the other hand, there are many indications of the actual protein nature of antibodies. For example, the protective and precipitating antibodies in anti-pneumococcus horse serum are more or less completely precipitated when the serum is added to 20 volumes of slightly acidulated water (15). About 90 per cent of the serum proteins remains in solution and 60 to 80 per cent of the pneumococcus antibodies is found in the precipitate and may be redissolved in saline and further purified. By removal of an inactive fraction with acid and treatment with zinc or aluminum salts metal-antibody compounds were obtained which were completely precipitable by the pneumococcus polysaccharide of the homologous type (16). Unfortunately, the globulin solutions remaining after removal of the zinc or aluminum were specifically precipitable to the extent of only 80 per cent. More recently, as a result of the quantitative study of the precipitin reaction discussed below, it has been possible to find a theoretical basis for dissociating certain pneumococcus polysaccharide-antibody precipitates with as mild a reagent as 10 to 20 per cent sodium chloride solution (17). In this way water-clear antibody solutions were obtained in which up to 100 per cent of the typical protein in the solutions was specifically reactive (cf. also (18)). When these solutions were prepared from antisera containing no preservative the rabbit antibody showed a sedimentation constant of 7×10^{-13} in the ultracentrifuge, while pneumococcus anti-carbohydrate in the horse showed a constant of 18×10^{-13} , both solutions being monodisperse (19). Thus antibody in the rabbit (antibodies to egg albumin were also studied) has the molecular weight of the principal globulin component of normal serum, while the dissociated pneumococcus anti-carbohydrate in the horse has a very much greater molecular size1 and apparently arises by elaboration of a small serum globulin com-

¹ Its molecular weight is 930,000; that of the rabbit antibody is 157,000. (Kabat, E. A., and Pedersen, K. O., Science, 87, 372 (1938).)

ponent which is present in the sera of all mammalian species, even that of the rabbit. These investigations furnish additional strong support for the protein nature of antibodies leading, as they have done, not only to the isolation of analytically pure antibodies, but also to an insight into the relationship of these substances to normal serum globulins.

There is, moreover, an additional mass of quantitative data supporting the protein nature of antibodies. Thus it has been shown that diphtheria toxin-antitoxin floccules consist mainly of denatured pseudoglobulin (20),—the serum protein fraction with which antitoxin is usually associated in the horse—and that the amount precipitated is independent of other serum proteins present or added. Other quantitative evidence will be discussed below.

If antibody is actually modified serum globulin, what is its relation to the antigen with which it reacts specifically, how is it formed, and how does it differ from normal serum globulin? In order to account for the specificity of antibody, Buchner assumed that antigen or antigen fragments actually entered into the antibody complex. This idea was abandoned by Ehrlich because of the large excess of antibody often produced. Although the Buchner hypothesis still has its adherents recent evidence against it is almost overwhelming, particularly the experiments with atoxyl azoprotein (Fig. 1, b). In this conjugated protein arsanilic acid (Fig. 1, c) is the hapten, or portion determining the specificity. If the specificity of the antibodies formed on injection of the azoprotein into animals depends upon the incorporation of specific antigen fragments into the antibody molecule these fragments would necessarily contain arsenic, and it should then be possible to detect arsenic in the antibody. However, as much as 30 ml. of antiserum contained no more than the faint traces of arsenic which are present in the same amount of normal serum (21). Similarly, the antibody to a deep red azoprotein, R-salt-azobiphenylazo-crystalline egg albumin (Fig. 2, d), was not red, as was the corresponding hapten, but colorless (22). The quantitative measurements discussed below also show that the actual amount of antibody formed with the aid of minimal quantities of antigen is so great as practically to preclude the participation of antigen fragments in the antibody.

If this be accepted and the theories of antibody formation involving incorporation of antigen into the antibody be eliminated, the theory which appears most reasonable to the chemist is the following (23). It is possible to conceive of normal serum globulin

synthesis as occurring in such a way that the spatial configuration of the cellular protoplasm impresses upon the synthesized globulin the spatial, chemical, and species-specific properties characteristic of the normal serum globulin of the animal in question. If the injected antigen or its partial degradation products should reach the sites of this synthesis the presence of the foreign protein could disturb the spatial relations which normally exist and distort them somewhat. It would be reasonable to suppose that this distortion would occur in a manner characteristic for the foreign material, so that when the new globulin, synthesized in this distorted manner, encountered the foreign protein once more in the circulation, or in vitro, interaction would be possible.

If antibodies differ from normal serum globulins only as postulated in this theory they would not be expected to show great chemical variation from normal globulin, since the differences

Table III

Mean Analyses of Normal and Antibody Globulin

Fraction	Horse serum globulin fractions including normal*	Toxin- antitoxin floccules*	Normal horse serum globulin (total)‡	Purified pneumo-coccus Type I antibody‡	Pneumococcus specific precipitates §	
					hydrate-anti-	Type II carbo- hydrate-anti- carbohydrate
Amide N	per cent 8.9 66.9	per cent 9.1 67.8	per cent 9.5 (filtrate Amino N) 59.2	per cent 9.2 59.4	per cent 4.5 75.2**	per cent 3.7 75.0**
Diamino N Cystine Tyrosine Tryptophane Carbohydrate	24.3 2.8 6.5 1.8 2.2-4.5	22.9 2.1 5.9 1.8 3.5†	(N) 1.1	(N) 1.3	2.6 5.5 2.1	3.1 5.5 2.2
Arginine N			9.0	8.8	Arginine 5.0	5.5
Histidine N			5.9	6.0	Histidine 1.0	1.1
Lysine N			8.3	10.0	Lysine 5.4	4.8
P					None 1.3 4.6 6.6	None 1.3 4.4 6.3

^{*} Hewitt, L. F., Biochem. J., 28, 2080 (1934).

[†] Antitoxin pseudoglobulin containing 20750 units per gm.

[‡] From Reference 24a.

[§] Calvery, H. O., J. Biol. Chem., 112, 167 (1935).

^{**} Amino N after hydrolysis.

would be mainly those of configuration or arrangement of the component amino acids. As will be seen from Table III, analyses of anti-pneumococcus specific precipitates and of diphtheria toxinantitoxin floccules fail to show marked differences from those of normal globulin, except that possibly strictly analogous fractions from normal serum have not been analyzed. The ultracentrifugal studies referred to above (19) also furnish evidence in favor of the theory. The only difference so far found with regularity is in the isoelectric point of pneumococcus anti-carbohydrate formed in the horse, this constant being at a more alkaline reaction than that of normal horse globulin (15, 16, 24a). However, a similar basic fraction occurs in normal globulin (24b) and recent cataphoresis experiments on highly purified solutions indicate a much more acid isoelectric point for antibodies (19a, 19c).

5. THE MECHANISM OF THE REACTION BETWEEN ANTIGEN AND ANTIBODY

Since the substances involved in immune reactions are, in general, colloids, the simplest way of accounting for such reactions as specific precipitation, agglutination, complement fixation or hemolysis, or toxin-antitoxin neutralization is to assume that oppositely charged colloidal particles combine to produce the observed effect. This view was championed by Bordet, who later modified it to the extent that immune reactions were considered adsorption phenomena. Ehrlich, however, maintained that actual chemical combination in definite proportions took place between antigen and antibody. In this he was supported by Arrhenius and Madsen, who drew further analogies with the more or less easily dissociated compounds between weak acids and weak bases.

While the colloidal theory offers a possible explanation of immune reactions it fails entirely to account for their specificity. If, for example, one adds to a rabbit anti-crystalline egg albumin serum a 1:10,000 solution of horse crystalline serum albumin, and to a rabbit anti-serum against crystalline horse serum albumin an equimolar solution of crystalline egg albumin, the sera, of course, remain clear. Both mixtures contain antibody globulin, presumably as the ionized sodium chloride compound, and if the sera are at the same pH the charges on the antibody particles would be the same, or nearly so, in both cases. As for the antigens, both are crystalline albumins with not very different isoelectric points, so that the charges on the particles of both must be of much the same order.

If the immune reaction depended, then, upon particle charges there should again be no evidence of change when some of the equally charged particles are taken from each mixture and added to the other. But in both mixtures horse serum albumin has thus been added to an anti-horse albumin serum and egg albumin to an anti-egg albumin serum, specific precipitation occurs, and the conclusion seems inescapable that the specific interaction is a chemical union. To draw a simple analogy from inorganic chemistry, there will of course, be no precipitate when sodium chloride is added to barium nitrate or sufficiently dilute sodium sulphate to silver nitrate, but when a portion of each solution is removed and added to the other, specific precipitation of barium sulphate and of silver chloride occurs in both mixtures.

Other analogies with inorganic ionic reactions have been pointed out (25a), and the participation of polar groups has again been emphasized (3, 24a).

6. THE PRECIPITIN REACTION

Of all immune reactions the precipitin reaction is relatively the simplest, but it has only recently been possible to overcome the analytical difficulties and subject the reaction to strict quantitative study (22, 25). Conditions for the maximum precipitation of antibody were first established with the aid of the nitrogen-free specific polysaccharide of Type III pneumococcus and with antibody purified according to (15) and containing 40 to 60 per cent of specifically precipitable protein (25a, f). The failure of nonspecific protein to influence the result was also shown (cf. also 26). This first absolute method for the determination of precipitin was then applied to Type I anti-pneumococcus sera (25b), in which a parallel was shown between the mouse-protection value and the maximum specifically precipitable antibody. The method was also found useful for the estimation of precipitin in anti-protein sera (25d) and for the determination of minute quantities of hapten or antigen (25c).

With the aid of the new quantitative method, starting again with the relatively simple nitrogen-free Type III pneumococcus hapten (S) and homologous antibody (A), the mechanism of the precipitin reaction was studied. By varying the proportions of the reactants it was possible to show that both components of the reaction are multivalent with respect to each other, and that their combination may be considered to take place in a series of competing

bimolecular reactions (25g). For example, the initial reaction between A and S would be:

$$A+S \rightleftharpoons AS$$
 (1)

In the region of excess antibody this would be followed by:

$$AS + A \rightleftharpoons AS \cdot A \quad \text{and} \\
AS + AS \rightleftharpoons AS \cdot AS$$
(2)

There would follow a third step, in which the competing bimolecular reactions involved would be:

$$\begin{array}{c} \text{AS} \cdot \text{A} + \text{A} \rightleftarrows \text{AS} \cdot \text{A} \cdot \text{A}, \\ \text{AS} \cdot \text{AS} + \text{A} \rightleftarrows \text{AS} \cdot \text{AS} \cdot \text{A}, \\ \text{AS} \cdot \text{A} + \text{AS} \cdot \text{A} \rightleftarrows \text{ASA} \cdot \text{ASA}, \\ \text{AS} \cdot \text{A} + \text{AS} \cdot \text{AS} \rightleftarrows \text{ASA} \cdot \text{ASAS}, & \text{and} \\ \text{AS} \cdot \text{AS} + \text{AS} \cdot \text{AS} \rightleftarrows \text{ASAS} \cdot \text{ASAS}, \end{array} \right\}$$

$$(3)$$

in which the first two reactions would occur only in the presence of enough A to carry the composition of the reaction product beyond the A₂S stage. Similarly, each compound formed in the third step would react with each other compound, or with more A, if present, to form still more complex substances and the reaction would continue until particles would be formed large enough to settle from the solution and precipitation would take place. This would doubtless be facilitated by the mutual discharge, with loss of affinity for water, of ionized groupings brought into juxtaposition by the series of chemical reactions (3, 25k, 1). Equilibrium in such reactions would necessarily lie far to the right, but the reversibility of the overall reaction has been amply demonstrated (25, 17, 19b).

If A and S are mixed in equivalent proportions the AS* formed in reaction (1) would merely polymerize in steps (2), (3) ... and the equivalence point (at which only minimal amounts of both components or none at all occur in the supernatant fluid) precipitate would be $(AS)_x$.

With more S maximum precipitation follows and free S remains. In the region of excess of S a similar series of expressions would apply, in which S and A would be interchanged. In addition, in the presence of a large excess of S, there is also an "inhibition zone" in which there is present a soluble compound, AS_y, contain-

^{*} More strictly, AmSn, since the molecular relationships are as yet unknown.

ing one more molecule of S in combination than the last insoluble compound (25a). Since this is formed only with a very large excess of S, all of the specific groupings of A would tend to react with S rather than with AS complexes, and there would be no large, intermolecular aggregates formed.

The final precipitate in all other cases would consist of antibody molecules held together by S molecules (cf. also (3)), which might be represented two-dimensionally as follows:

The mathematical treatment of the entire course of the reaction involves certain simplifying assumptions. For details the reader is referred to reference (25g), from which much of this section of the chapter is quoted or paraphrased. Under the assumption that the mass law is applicable it is possible to derive the following expression in the region of excess antibody for steps (1) and (2) which is as far as it is practically necessary to calculate the reaction:

mg. antibody N pptd. =
$$2RS - \frac{R^2S^2}{A}$$
 (4)

in which R equals the antibody N to S ratio at the beginning of the equivalence zone (where antibody just ceases to be in excess), and A equals the amount of antibody N precipitated at this point. Excellent agreement is obtained between the values actually found and those calculated according to equation (4).

A relationship useful in its application to unknown sera may be derived from equation (4). If both sides of the equation be divided by S, the resulting equation,

$$\frac{N}{S} = 2R - \frac{R^2}{A}S\tag{5}$$

is that of a straight line. Thus, if the values of the ratio found in the region of excess antibody are plotted as ordinates against the amounts of S added as abscissae, a straight line is obtained. The intercept on the y axis gives the value of 2R, while the slope is $-R^2/A$, from which A may be calculated.

Equations (4) and (5) have been found to hold for a number of protein-anti-protein systems as well (25h, i, k, l) and as a result it is now quite generally possible to characterize an anti-serum or anti-body solution in the region of antibody excess by as little as two analyses of the washed specific precipitate for nitrogen, in duplicate, from which the line according to equation (5), may be drawn. A similar equation holds in the region of excess of S while in the protein-anti-protein systems the inhibition zone with excess of antigen may follow closely on the equivalence zone. In the inhibition zone, in the case of the S-A and dye-anti-dye systems (25g, h) it is possible to calculate the amount of precipitate in the inhibition zone also with the aid of an apparent dissociation constant for the soluble compound or compounds formed in this zone.

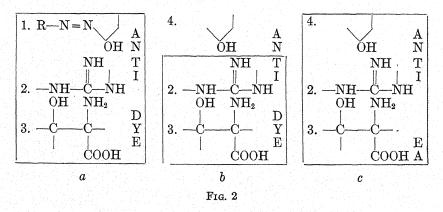
In all of the above it is to be noted that the composition of the precipitate depends, not on the concentration of antigen or antibody, but on the relative proportions in which they are mixed, and this is reflected in the equations given, in the derivation of which the volume factors cancelled. In the derivation, also, it was assumed that antibody behaves statistically as if it were a single substance. It is known, however, that antibody formed even in response to injections of a single, definite chemical substance is not uniform, but consists of portions of varying reactivity, so that the relations found above are to this extent approximations.

With their aid, however, anti-sera may be quite accurately characterized, in some instances over their entire reaction range from excess antibody to excess antigen, by means of a few micro-determinations for nitrogen. Once the equations for a serum have been established, the amount of antibody nitrogen precipitated by any amount of hapten or antigen may be calculated in absolute terms, that is, mg. per ml., and not in the relative measures hitherto used. Moreover, immunological puzzles such as the coexistence of antigen and antibody in body fluids and the Danysz phenomenon also find their explanation on the basis of this work (6b, c). The process of immunization in animals may also be followed quantitatively at its various stages and the progressive changes involved accurately mapped out (25i).

The use of the quantitative precipitin method has also led to the recognition of sharp differences between at least a few instances of cross precipitation and the homologous reactions, and to a clear, simple explanation of these examples. Thus it was found (25e) that rigorously purified R-salt-azobiphenylazo-crystalline egg al-

bumin (Fig. 1, d), precipitated only in occasional anti-egg albumin sera. Nevertheless, the reverse reaction occurred, suitable amounts of crystalline egg albumin precipitating anti-sera obtained on injection of the purified dve protein. In some anti-dye sera nearly all of the antibody could be precipitated with very large amounts of egg albumin, amounts far greater than sufficient to throw the homologous reaction into the inhibition zone. Thus the form and direction of the reaction curve differed from that characteristic for homologous reactions. In certain anti-dye sera very small amounts of egg albumin failed to cause precipitation, although larger quantities did. At all points free egg albumin could be detected by the addition of anti-egg albumin, so that the cross precipitation was most easily explained on the basis of a loose, highly dissociated combination between the egg albumin and anti-dye, as opposed to the firm, almost undissociated union between dye-protein and antidye, or egg albumin and anti-egg albumin.

This may, perhaps, be more easily visualized in the following diagrams (Fig. 2):



Grouping $1,^2$ in accordance with Landsteiner's findings, is the dominant antigenic group in the dye-protein, while 2 and 3 are other arbitrarily chosen groups of which the steric configuration is characteristic for egg albumin. The dominant reactive group on the anti-dye molecule would be one evoked in the animal by virtue of the dominant antigenic group. The resulting dye-anti-dye combination might therefore be represented by diagram a, and would

² It is not certain that this grouping is actually the dominant antigenic grouping of the dye since specific precipitation is not inhibited by the R-salt portion of the molecule.

be expected to be relatively firm and undissociated. Other less important groups on the antibody molecule might be expected to result from the groupings 2 and 3, characteristic of the egg albumin portion of the dye molecule. Thus crystalline egg albumin, with its dominant antigenic group 4, arbitrarily chosen by analogy with 1, might be expected to react with the anti-dye by virtue of groupings 2 and 3. The union could reasonably be postulated as a weak, highly dissociated one, since there would be no anti-4 in the anti-dye, and egg albumin contains no grouping 1 to react by virtue of the anti-1 present (Fig. 2 b). Again, crystalline egg albumin, with its dominant antigenic group 4, would be expected to form the observed firm, undissociated union with anti-egg albumin which contains anti-4, as represented in Fig. 2 c.

While these views are applicable without change to additional instances of cross precipitation, they must be modified in others, for it is now known that in many cases, even that of the single crystalline antigen, egg albumin, several independently reactive antibodies are formed (25g, h, i), and that only a portion of these may cross-react (cf. also (27)).

7. BACTERIAL AGGLUTINATION

When a bacterial suspension is treated with a suitable dilution of an anti-serum obtained by injection of an animal with the homologous bacterium, antibody is deposited on the bacteria. These clump together and are then said to be "agglutinated." The test is an exceedingly sensitive one for antibody, and is, in many instances, more delicate than the precipitin reaction or even any of the usual direct chemical tests for protein.

Bacterial agglutination may be treated chemically in much the same manner as the precipitin reaction. There is a close parallel between the successive stages of the precipitin reaction and the agglutination of type-specific pneumococci (28). Thus bacterial agglutination, as also fore-shadowed by earlier work (29), is merely a precipitin reaction at the surface of bacteria, and, with this restriction, is governed by the same laws. By the use of a washed bacterial suspension containing a known amount of nitrogen, agglutinins may be determined in certain anti-sera by the increase in the amount of nitrogen after agglutination, centrifugation, and washing of the suspension, provided the suspension is in excess and the increase in nitrogen is large enough to be within the range of the micro-Kjeldahl method (30). The quantitative correspondence and

identity of precipitin and agglutinin have thus been shown in a type-specific anti-pneumococcus serum.

The above absolute method for the estimation of agglutinin has led to the formulation of a quantitative theory of bacterial agglutination based on the same considerations as for the precipitin reaction and leading to equations of the same form (31). These have been found to describe accurately the agglutinating behavior of Type I anti-pneumococcus serum. The theory also postulates the building up of large aggregates (agglutination) by chemical interaction of multivalent antibody on the surface of bacteria with multivalent specific polysaccharide on the surface of other bacteria, and this was demonstrated experimentally. The flocculating action of electrolyte, by which agglutination was explained in the older theories, as well as potential and cohesive force, are thus shown to be of minor influence (cf. also (32)).

8. TOXIN-ANTITOXIN NEUTRALIZATION

The toxin-antitoxin reaction, which also involves the direct union of antigen and antibody, is thus very similar to the precipitin reaction and agglutination. It is, indeed, often accompanied by flocculation of the toxin-antitoxin complex, particularly in the range approaching neutrality. The chief difficulties in the way of a chemical study of the reaction have been the lack of chemical knowledge of toxins and the cumbersome mechanism of the animal tests for toxin and antitoxin.

The introduction, as in the precipitin reaction, of quantitative nitrogen estimations on the floccules produced with varying proportions of toxin (T) and antitoxin (A) has shown that precipitation takes place in the range covered by the relative proportions TA to TA₂(33). With highly purified toxin, the quantitative method, in correlation with animal tests, has permitted an even closer analysis (34). In the flocculation zone it was found that all of T and A added were precipitated, indicating an equivalence zone much as in the precipitin reaction. From the mean nitrogen ratios of the components at the most rapid flocculation point and the amount of nitrogen precipitated it was calculated that 1 L, dose of toxin = 0.00046 mg. and that 1 unit of A = 0.0016 mg. N for native antitoxin of normal in vivo: in vitro ratio. A single nitrogen determination therefore permits the calculation of both T and A in unknowns. In this work a three-fold combining range was indicated in the flocculation, or equivalence zone, with the possibility of other compounds in solution on either side of the zone. The above studies confirm earlier predictions (35, 6b, c) and establish the analogy with the precipitin reaction.

9. COMPLEMENT FIXATION AND IMMUNE HEMOLYSIS

When red cells of another species are injected into an animal, antibodies are produced which agglutinate or dissolve cells of the kind injected and are therefore termed hemagglutinins or hemolysins. Complement, a complex and labile constituent of most fresh, native sera, is required for the lysis, and as the reaction is an easy one to observe, it furnishes a convenient indication for the presence or absence of complement. Thus, in the Wassermann reaction for syphilis the "lipid antigen" is added with complement to a suspected serum in which the complement present has previously been "inactivated." If the serum is syphilitic and complement is used up in the reaction between the antigen and antibodies or "reagins" present, a mixture of red cells and hemolysin, when subsequently added, will not be hemolyzed, and a "4+ positive Wassermann" is reported. Much as in the case of the agglutinin reaction, it has been shown that the "lipid antigen" suspension removes more nitrogen from a syphilitic serum than a normal one, so that the so-called "reagin" is probably much like an antibody, if not actually one (36).

10. ALLERGY AND ANAPHYLAXIS

"Allergy" refers to an altered state in which an animal becomes locally or wholly sensitive to an antigen. If the sensitized animal shows alarming or fatal generalized symptoms on re-injection of the homologous antigen or hapten the phenomenon is called "anaphylaxis." While the manifestations of allergy and anaphylaxis are undoubtedly due to a primary union of antigen and antibody there is some evidence that substances liberated in the tissues as a result of this action may be the direct cause of the symptoms. For a full discussion see (37).

³ For work on the nature of the Wassermann "antigen" see Fitzgerald, J. G., and Leathes, J. B., Univ. of Calif. Pub. Path., 2, 39 (1912); Schmidt, C. L. A., and Coffey, S. E., J. Infect. Dis., 32, 119 (1923); Grüneberg, T., Klin. Wochschr., 15, 1263 (1936); Fischer, Ö., Steinert, J., Fischer-Dallmann, R., Z. Immunitätsfrsch., 89, 133 (1936); Steinert, J., Z. Immunitätsfrsch., 89, 139 (1936); Ornstein, I., Dragos, M., and Muhlberg, S., Compt. rend. Soc. biol., 124, 398 (1937). A review of the technics is given by Gershenfeld, L., Amer. J. Pharm., 109, 468 (1937). See also Eagle, H., J. Lab. Clin. Med., 22, 300 (1936).

For some time evidence has been accumulating that animals can be made sensitive to relatively simple substances, which by themselves are ordinarily incapable of eliciting any immune response and can only act in the sense of haptens (38). Many instances of drug allergy appear to be of this nature, and the simplest explanation at present would seem to be that in certain individuals conjugation of the drug or hapten takes place with body protein, forming a new antigen which gives rise to the sensitization phenomena.

11. RÉSUMÉ

In this chapter the writer has attempted a brief survey of present-day chemical knowledge regarding antigens, antibodies, and their interaction, with especial reference to protein chemistry. It must be obvious that the state of chemical knowledge of the complex field of immunity is fragmentary and unsatisfactory, but that a beginning has been made sufficient to emphasize the essentially chemical and ultimately minutely determinable basis of biological specificity. Strong evidence has been presented of the chemical union of antigen with antibody in multiple proportions, and of the possibility of expressing this union in terms of the laws of classical chemistry. New and absolute quantitative micro-methods have been worked out and these should be valuable aids in keeping pace with the broadening knowledge of the fine structure of the proteins toward a final complete understanding of immune processes.

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CHAPTER XVIII

THE RÔLE OF PROTEINS IN NUTRITION

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1. THE NEED FOR PROTEIN

Protein is a component of every living cell. It follows, therefore, that all living forms must have a supply of nitrogen available. In the case of green plants, this demand for nitrogen may be satisfied by the inorganic ammonium and nitrate salts. Some bacteria as well as a few green plants are able to utilize atmospheric nitrogen. Manifestly, these several organisms are provided with a chemical mechanism or set of mechanisms whereby nitrogen which is received in its simple inorganic forms can be synthesized to protein with its various constituent amino acids. Such, however, is not the case with the higher members of the animal kingdom and it is to the nitrogen requirements of these that attention is directed in this chapter.

About a hundred years ago, the idea of a universal food or aliment for animals was discarded in favor of the view that the principal organic ingredients of the diet might be classified on the basis of what we now term carbohydrate, fat, and protein. Protein was accorded the importance of being an indispensable requirement of the animal organism. The theory elaborated at that time naturally involved certain misconceptions. However, the central idea, that a supply of protein or its component structures is in constant demand by the higher animal organism, has subsequently been supported by a great body of experimental work and has long since been accepted as beyond reasonable challenge.

It is not within the scope of this discussion to trace the gradual development of our present views with respect to the fate of protein in the alimentary tract of the animal and the state in which this protein is absorbed and distributed in satisfying the needs of the body.¹ For our purpose, it must suffice to point out that ingested

¹ For an extensive review of the literature on this subject, the reader is referred to Catheart (1), and further for a résumé of the early theories concerning food and foodstuffs to Mendel (2).

proteins are not utilized directly in their native form or even after only slight alteration, but are subjected to the action of the several proteolytic enzymes in the alimentary tract and are split to amino acids. These are absorbed and are circulated by the blood stream. This view in its entirety was adopted only after it was demonstrated that amino acids occur normally in the blood and that they increase, as expected, during the absorption of protein (3. 4, 5). The complete, or relatively complete, hydrolysis of proteins in the process of assimilation entails, of course, that the ingested proteins lose their identity and that the demand of the body for this class of compounds is actually resolved into a demand for some or all of the amino acids which are produced by the hydrolysis of proteins. Thus, the some twenty or more amino acids (see Chapter II) which are derived from proteins may be viewed as the real mediums of exchange in the transfer of protein from the tissues of plants and animals to the tissues of the higher animals. This mechanism enables each organism to make its own selection and arrangement of amino acids in the synthesis of its particular proteins. These are structurally different from the hundreds of foreign proteins present in the wide variety of foods which are included in the normal diet. The mechanism may also be considered to be a protection against the allergic and anaphylactic effects of foreign proteins which otherwise might be introduced into the body in their native states.

2. THE NEED FOR AMINO ACIDS. METHODS OF INVESTIGATION

The realization that the several amino acids are the units which are ultimately employed by the body naturally raised the question as to whether all or only some of these are required and, in the event that not all are required, the further question as to which can be synthesized and which cannot be synthesized by the body. The extensive experiments which have been carried out to elucidate this problem comprise one of the chapters in the intriguing story of the search for the manifold dietary factors which are essential to life.

Various methods of attack have been employed in studies of amino acid requirements. One method involves the preparation and feeding to a growing animal of a diet nutritionally adequate

² There are apparently no quantitative experimental data proving that amino acids are the only form in which proteins are absorbed. Indeed, allergic reactions from food and enteral immunization are explained on the basis of the absorption of *traces* of unaltered protein from the gut.

with respect to fat, carbohydrate, inorganic salts, vitamins, and all of the amino acids except the one which is being tested. If these conditions are met satisfactorily, then the absence of an indispensable amino acid results in failure of growth, whereas restoration of the missing factor in pure form promotes growth. However, if a dispensable amino acid is omitted from the diet, growth of the animal is not affected. Growth is measured in such terms as body weight or body length. Another method of approach is similar to the one iust described but differs from it in that the quality of the amino acid intake is judged by the criterion of nitrogen balance rather than by that of change in body weight. A negative nitrogen balance like the loss of body weight, indicates that there is nutritive failure and that the amino acid which is missing from the diet is indeed essential. This conclusion is confirmed and strengthened by the finding that a dietary supplement of the amino acid results in a positive nitrogen balance thus indicating retention of nitrogen in the tissues of the body.

The greatest difficulty in such experiments has been that of eliminating a given amino acid without otherwise interfering seriously with the quality of the diet. At times it is possible to omit a particular amino acid by feeding a protein which by nature possesses the appropriate deficiency. Thus, a limiting factor of the protein, gliadin, is its low content of lysine. There are other similar cases, but in general the observed instances of such specific clearcut deficiences have not proved sufficiently numerous to permit an exhaustive study of amino acid requirements. However, it has been demonstrated, as might be expected from our previous discussion of the fate of proteins in the alimentary tract, that an amino acid mixture, secured by appropriate hydrolysis of a protein of satisfactory nutritive properties, e.g., casein, serves nutritionally in place of the original protein (6). Such a mixture of amino acids may be made deficient in one or more of these units by subjecting the mixture to suitably selective fractionation procedures. Here again, the number of available deficient amino acid combinations has been limited due to the lack of specific reagents and methods. Inasmuch as a mixture of amino acids can be substituted satisfactorily for protein in the diet, it must appear that the ideal way of studying amino acid requirements would be to feed combinations of the pure crystalline amino acids. Indeed several attempts have been made in this direction but, due to the fact, as we now know, that all of the indispensable amino acids were not incorporated in the diet, most of these experiments were destined to fail. Recently reported successful studies of this kind will be discussed presently.

Besides the problem of arranging the main nitrogenous fraction of the ration so as to have the desired deficiency, there is the further one of insuring against contributions of the missing amino acid from other essential articles of the diet. Fat, vitamins A. D. and E. carbohydrate and mineral salts which are free or relatively free of amino acids are quite available. However, if yeast, which is commonly employed experimentally as a source of the vitamin B factors, is fed as the only source of protein, very good growth results. Hence yeast, even in limited quantity, must supply some of each essential amino acid. The difficulty has been largely overcome by the use of limited amounts of yeast or by the use of extracts of yeast or other materials which are potent in the vitamin B factors. In consequence of the great obstacles which have been encountered in eliminating entirely a given amino acid from the diet, the successful biological demonstrations of the indispensability of certain amino acids have, in general, been much more significant than the negative indications with respect to others.

The uncertainties that have attended the results with the apparently dispensable amino acids have directed attention to other methods of securing information. A limited number of the amino acids are conjugated with foreign organic compounds in the body and are excreted in the urine (see Chapter V). Demonstration that the amount of an amino acid which is excreted in conjugated form is greater than the amount of the available preformed amino acid constitutes evidence that it is dispensable. Still another method of showing that an amino acid may be synthesized in vivo is to prove. by comparative analysis, that the total increment of the amino acid in the body is significantly greater than the dietary intake during the corresponding period. Both of these last mentioned methods are, of course, subject to any errors or inadequacies involved in two sets of analyses. Of all the procedures for determining the amino acid requirements, the one which employs growth as a criterion, particularly in the case of the rat, has yielded most of our information.

3. EVIDENCE FOR THE DISPENSABILITY OR INDISPENSABILITY OF THE INDIVIDUAL AMINO ACIDS

(1) Lysine and Tryptophane. In one of the very early attempts to ascertain the need of the animal for particular amino acids,

Willcock and Hopkins (7) showed that a diet containing zein, which lacks lysine and tryptophane, as the source of protein did not support growth of young mice or even permit maintenance of body weight. Addition of tryptophane to the diet did not greatly affect loss of weight but did enable the animals to maintain life longer than with zein alone. These observations, pointing to the nutritive importance of tryptophane, were followed a few years later by the beautifully incisive experiments of Osborne and Mendel. These investigators employed growing rats as the test animals. They were fed various purified proteins as the sources of nitrogen in the basal

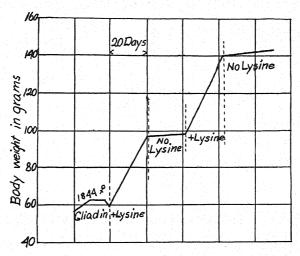


Fig. 1. Indispensability of lysine for growth. (Osborne, T. B., and Mendel, L. B., J. Biol. Chem., 17, 342 (1914).)

diets. It was shown (8) that when gliadin was fed the rats did not grow. However, on supplementing gliadin with lysine growth was resumed (see Fig. 1). Lysine is, therefore, an essential amino acid. Abderhalden (6, 9, 10) performed experiments on dogs which were fed a diet containing a casein hydrolysate from which the tryptophane had been removed. The dogs lost weight and were in negative nitrogen balance. When tryptophane was added to the diet the nitrogen balance of the animals improved and there was a gain in body weight. Osborne and Mendel were able to show by experiments similar to those with gliadin and lysine that the combination of lysine and tryptophane supplements zein in the promotion of growth. Neither lysine nor tryptophane singly is sufficient to accomplish this result and zein itself, as has been stated, does not

prevent loss of body weight (see Fig. 2). These and comparable observations from many different laboratories leave no doubt as to the indispensable nature of lysine and of tryptophane.

(2) Histidine and Arginine. Histidine is an essential component of the diet, as was discovered in studies on the growth-promoting properties of the amino acids from casein following the removal of the histidine and arginine. The resulting mixture was found to be inadequate for maintenance, but the addition of either histidine or arginine was claimed to cause an improvement in body weight

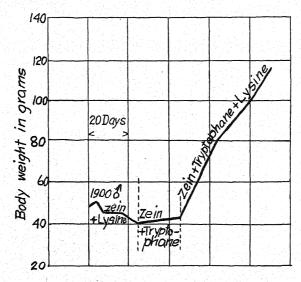


Fig. 2. Essential nature of both lysine and tryptophane in growth. (Osborne, T. B., and Mendel, L. B., J. Biol. Chem., 17, 347 (1914).)

or a resumption of growth in the test animals (11). The implied metabolic relationship of the two amino acids was rationalized on the basis of their somewhat suggestive similarities in structure. Exhaustive repetitions of these experiments have amply confirmed the evidence for the essential nature of histidine, but have entirely failed to show that arginine can replace histidine in the diet. The growth of the test animals was found to depend entirely on the addition of histidine and, in no case, on that of arginine (12). On the basis of these and similar studies, histidine is to be regarded as indispensable. The status of arginine, however, remained uncertain. The fact that large amounts of arginine could be removed from an adequate mixture of amino acids without interfering with

its growth-promoting properties was favorable to the view that arginine is a non-essential component of the diet (13). This view was greatly strengthened by the finding (14) that it is possible for growing rats to accumulate in their tissues two or three times as much extra arginine as is contained in the total diet ingested during the experiment. Subsequently, Rose (cf. 14a), employing synthetic mixtures of the amino acids devoid of arginine, has demonstrated

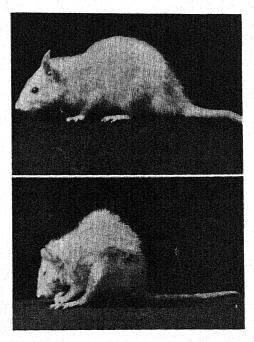


Fig. 3. Effect of a deficiency of histidine. For about 3 months both animals were given essentially the same diet except that the animal shown in the upper part of the photograph received a supplement of histidine.

(Rose, W. C., and Cox, G. J., J. Biol. Chem., 61, 761 (1924).)

that rats can synthesize sufficient arginine to provide for growth at about 70 to 80 per cent of the normal rate. A dietary supplement of the amino acid is necessary, however, if maximum growth is to be attained. Other investigators (14b) have found that arginine may be a limiting factor in the growth of chicks notwithstanding the considerable arginine content of the basal diet employed.

The effect of a histidine-deficient diet is shown in Fig. 3.

(3) Cystine and Methionine. Among the several important contributions of Osborne and Mendel to the knowledge of the physiology of amino acids was the demonstration that, if a diet contain-

ing a *limited* amount of casein is fed to the young rat, growth is curtailed, but that a dietary supplement of cystine leads to a resumption of growth (15). This discovery has been confirmed many times not only with casein but with other proteins or protein mixtures. Further indication of the requirement of cystine has been secured by nitrogen balance experiments (16) and by study of the limitations of the ability of the animal to provide cystine for conjugation with bromobenzene in the production of bromophenylmercapturic acid (17).

The conclusion that cystine is an essential dietary factor appeared to be established beyond question. The idea appeared for many years to be perfectly logical on the grounds that cystine was the only sulfur-containing amino acid known to be present in proteins. However, following the isolation of a second sulfurcontaining amino acid, methionine, it was discovered (18) that methionine as well as cystine will unmistakably stimulate growth of animals on diets heretofore called cystine-deficient. Further evidence of a relationship between the two amino acids is to be found in experiments showing that the administration of methionine increases the excretion of cystine in a cystinuric patient (19); and that cystine and methionine both give rise, in a specific manner to an increase in the urinary organic sulfur excreted by the animal poisoned with bromobenzene (20). Since the basal diets employed in the growth experiments are known to contain both cystine and methionine, the explanation of growth stimulation by either amino acid appeared to lie either in some type of actual interconversion of the two amino acids or in some particular essential requirement of sulfur which is satisfied by both amino acids. The need of studies of the supplementing value of cystine and methionine in connection with basal diets entirely devoid of both amino acids was thus indicated. Recent feeding experiments (21, 21a), with diets containing mixtures of pure amino acids, show that methionine is indispensable, and that when the intake of the two amino acids is restricted to minimal amounts, the addition of cystine alone does not enhance the value of the regimen. These findings, of course, are compatible with the idea that cystine may satisfy a part of the sulfur-containing amino acid requirement for growth provided that the intake of methionine is only partially restricted.

(4) Threonine. (α -amino- β -hydroxy-n-butyric acid.) It has been known for some time that the mixture of amino acids secured

by hydrolyzing a nutritionally satisfactory protein (casein, for example), when incorporated in an otherwise adequate diet, would, like the native protein, support the growth of a young animal. On the other hand, it had not been demonstrated in well controlled experiments that a synthetic mixture of most or all of the available known amino acids was equivalent in this respect to the crude mixture of hydrolytic products of casein. In fact, a thorough investigation (22) of this point showed that a mixture of 19 of the amino acids commonly occurring in proteins does not permit growth or even uniform maintenance of body weight. These results seemed best explained on the basis that there remained some undiscovered essential factor, probably an amino acid. Proceeding along this line, Rose and his students (23, 24, 25, 26) carried out an extensive and exhaustive program of experiments to determine inter alia the identity of the "missing link." It proved to be α -amino- β -hydroxyn-butyric acid (threonine). A diet containing a mixture of threonine and nineteen other amino acids, as listed in Table I, was found

Table I

Amino Acids, a Mixture of Which May Replace Dietary

Protein in Supporting Growth*

Alanine	Lysine
Arginine	Methionine
Aspartic acid	Norleucine
Cystine	Phenylalanine
Glutamic acid	Proline
Glycine	Serine
Histidine	Threonine
Hydroxyproline	Tryptophane
Isoleucine	Tyrosine
Leucine	Valine

^{*} Not all of these amino acids are required as is discussed subsequently.

to support excellent growth. Omission of threonine occasioned a loss in body weight.³ These results, which are illustrated in Fig. 4, p. 984, constitute a beautiful and, it may be noted, the first satisfactory demonstration that a synthetic mixture of purified amino acids can replace protein in the diet of the growing animal. Obviously, this discovery has opened the way to critical investigations of the requirements of all of the amino acids and a great many problems related to the physiology of amino acids and proteins.

³ Wood, M. L., Madden, R. J., and Carter, H. E., J. Biol. Chem., 117, 1 (1937), have described a method for synthesizing a mixture of α -amino- β -hydroxy-n-butyric acids which supports the growth of rats when the mixture is included in the diet in amounts of 2.5 to 4 per cent.

(5) Glycine. Glycine has long been regarded as a non-essential component of the diet. Certain proteins found by chemical analysis to be deficient or possibly devoid of glycine are, or can be made, adequate for growth without the addition of glycine. This type of evidence, however, is provisional, being qualified by the difficulties of making accurate determinations of small amounts of glycine in the dietary protein. On the other hand, numerous investigations⁴

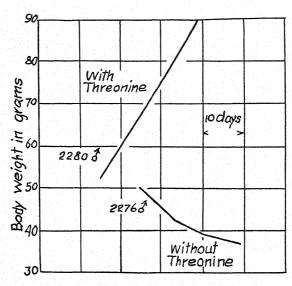


Fig. 4. Growth on diets containing in place of protein a mixture of 20 (cf. Table I) highly purified amino acids including threonine; and failure of growth with the single omission of the indispensable threonine.

(McCoy, R. H., Meyer, C. E., and Rose, W. C., J. Biol. Chem., 112, 291 (1935).)

have been recorded of the excretion of hippuric acid produced in animals by conjugation of administered benzoic acid with glycine available from dietary or metabolic sources. The results indicate that large but not unlimited amounts of glycine may be excreted as hippuric acid, that the glycine available from the diet or from the tissues of the animal hardly seems sufficient to account for the amounts which are excreted, and finally, that glycine can originate from nitrogenous sources, the end products of which would otherwise be excreted as urea. Such results may be conservatively considered as quite favorable to the view that glycine is not essential—a view now confirmed by successful growth experiments with

⁴ For a more detailed discussion and for citations to the literature the reader is referred to articles by Griffith and Lewis (27) and Griffith (28).

mixtures of pure amino acids from which glycine is omitted (26a).

- (6) Tyrosine and Phenylalanine. The opinion recurrently expressed in the literature (9, 10, 29) that tyrosine is required by the animal or that the needs for tyrosine and phenylalanine, which are closely related structurally, are met by either amino acid alone. has not been substantiated. No restriction of growth in rats was found with diets which contained protein hydrolysates either low in tyrosine or treated in a variety of ways to remove or to destroy the tyrosine (30, 31, 32). Womack and Rose (33), employing a diet containing a series of pure amino acids supplemented with a partially purified fraction of threonine, have demonstrated in a convincing manner that phenylalanine is essential to growth. They have also contributed further data showing that good growth is possible with a very limited intake of tyrosine. Tyrosine cannot replace phenylalanine, but whether phenylalanine can be converted to tyrosine in the normal intact organism remains to be seen. The fact that small amounts of tyrosine accumulate during the perfusion of the surviving liver with blood containing added phenylalanine (34) has been regarded as suggestive of such a relationship.
- (7) The Dicarboxylic Acids, Proline and Hydroxyproline. Protein hydrolysates from which glutamic and aspartic acids have largely been removed by precipitation procedures, support good growth and are not enhanced in this respect by supplements of these amino acids (35, 13). Another indication of the probable non-essential nature of glutamic acid is had in the finding (36) that when phenylacetic acid is administered to man it is conjugated with the amide of glutamic acid, glutamine, at the expense of nitrogen which otherwise would be excreted as urea. β -Hydroxyglutamic acid, thought to be dispensable on the grounds that none could be detected in edestin (37), which protein nevertheless supports growth, has now been demonstrated to be non-essential. β-Hydroxyglutamic acid may be omitted from a mixture of amino acids which satisfies the requirements for excellent growth (26).

Contrary to unconvincing claims that proline is essential for growth and that hydroxyproline and proline are interchangeable with respect to the dietary requirement, growth with protein hydrolysates so extracted as to remove most, if not all, of the proline speaks for the dispensable nature of this amino acid (38). Latterly, Rose (cf. 14a) has reported conclusive evidence for the dietary dispensability of proline, hydroxyproline, and all three dicarboxylic acids.

(8) Other Amino Acids. On the basis of further feeding experiments with purified diets, Rose and his associates have found that leucine and isoleucine (39) as well as valine (21, 14a) are essential amino acids, whereas serine (26a) and alanine (39a) are dispensable. Table II, compiled by Rose (14a), is a summary of our present information with respect to the requirements of the growing animal for amino acids.⁵

Table II

Final classification of the amino acids with respect to their growth effects

Indispensable	Dispensable
Lysine	Glycine
Tryptophane	Alanine
Histidine	Serine
Phenylalanine	Norleucine
Leucine	Aspartic acid
Isoleucine	Glutamic acid
Threonine	Hydroxyglutamic acid
Methionine	Proline
Valine	Hydroxyproline
*Arginine	Citrulline
	Tyrosine
	Cystine

^{*} Arginine can be synthesized by the animal organism, but not at a sufficiently rapid rate to meet the demands of *normal* growth.

Note.—For previous reviews of amino acid requirements, the reader is referred to Mitchell and Hamilton (40) and to Rose (41, 42, 42a) and especially to Rose (14a).

4. THE NUTRITIVE RÔLE AND VALUE OF PROTEINS IN THE DIET

While extremely important advances in studies of the nature of protein requirement have been accomplished by feeding experiments with the *free* amino acids, the fact remains that the higher animal organisms have depended for countless generations in the past and still must depend on native proteins as their main source of nitrogen and of amino acids. Consequently, it is of great practical importance to ascertain whether all proteins have the same nutritional value and, if not, which particular proteins or combinations of proteins suffice to meet the various needs for this type of foodstuff. As has been emphasized in the previous discussion, the

⁵ Corley, R. C., Wolf, P. A., and Nielsen, E. K., *Proc. Amer. Soc. Biol. Chem.*, xxvi (1938) (*J. Biol. Chem.*, 123 (1938)), have reported that histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine are apparently indispensable for the maintenance of nitrogen balance in the adult albino rat.

value of protein must depend on the kinds and amounts of amino acids made available to the animal through the processes of digestion and absorption. In particular, any protein which is to be considered complete in the nutritional sense must supply not less than a certain minimum of each and every indispensable amino acid.

Inspection of the tables (cf. Chapter IV) showing the amino acids recovered from the hydrolytic products of various proteins, immediately reveals the fact that different proteins exhibit diverse values for the several units contained in the molecule. This is true for those amino acids which are known to be essential as well as for those considered not to be necessary. Hence, it is to be expected

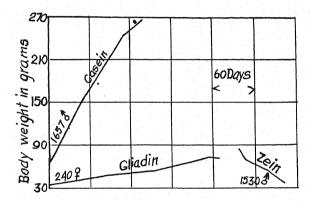


Fig. 5. Typical graphs of growth on foods containing single proteins. Proteins are not equivalent in supplying needs of the organism.

(Mendel, L. B., J. Amer. Med. Assn., 64, 1542 (1915).)

that the nutritional values of individual proteins will in like manner vary widely. This has been borne out by physiological studies of a large number of purified proteins. For example, casein and edestin, when incorporated in diets to the extent of about 18 per cent, support good growth. Gliadin, which is deficient in lysine, provides an amino acid supply sufficient only for approximate maintenance of body weight, whereas zein, which is deficient in tryptophane and lysine, and gelatin, which is lacking in several of the amino acids, are inadequate even for purposes of maintaining body weight (see Fig. 5). Suitable supplements of pure amino acids have been shown to improve greatly the nutritional value of these and other inadequate proteins.

Not only may pure amino acids supplement deficient proteins, but pure proteins may supplement each other as exemplified in Fig. 6. The efficiency of a diet, therefore, with respect to its content of proteins or amino acids depends on the total amounts of the indispensable amino acids available from the different proteins or protein derivatives present in all of the food articles making up the diet. This fact, so admirably demonstrated in the great difficulties met in attempting to prepare diets absolutely devoid of a given amino acid for experimental work, doubtless provides a con-

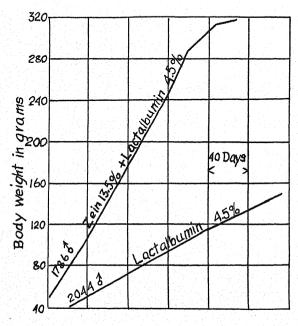


Fig. 6. The supplementing effect of proteins in promoting growth. Growth on the diet containing 4.5 per cent of protein as lactalbumin is much restricted, and loss of weight ensues when zein is the sole protein of the food.

(Mendel, L. B., J. Amer. Med. Assn., 64, 1544 (1915).)

siderable factor of safety against the occurrence of an extreme dietary deficiency in any one indispensable amino acid in the naturally chosen food of man and animals. Obviously, such a marked specific deficiency in one amino acid is not to be confused with the general amino acid deficiency which supervenes in starvation or in any dietary régime involving a definitely limited intake of protein. The minimal and optimal amounts of protein required by the organism have been much studied, and the daily requirement for man has been estimated to be from 50 to more than 100 gm. per day. Such estimates naturally have meaning only in terms of mixed proteins with some sort of an average content

of indispensable amino acids or in terms of a particular designated group of proteins of high nutritional value.

5. EFFECT OF HEAT TREATMENT ON THE NUTRITIONAL VALUE OF PROTEIN

Cooking has generally been supposed not to exert any marked effect on the utilization of protein by the animal organism. Data reported in this connection have been conflicting, though in specific instances cooking seems to improve the digestibility of the protein. In recent years, the question has been studied more intensively. and it has been shown, for example, in one study (43) that, following heating or toasting at temperatures of 150° to 200°, several cereals, wheat gluten, and casein support less vigorous growth and less favorable nitrogen retention than the respective materials which had not been subjected to the heat treatment. The digestibilities of the heated materials were found to be unaltered although supplements of lysine and histidine were found (44) to increase the nutritional value of heated casein but not that of the unheated casein. That the detrimental effect is not confined to dry heating is illustrated in further experiments (45) showing that cooking with water lowers the value of the protein of raw beef. The severity of the treatment employed apparently is a factor. Other investigators (46) find that the loss in nutritive value of the proteins of beef liver. by heating at 120°, is to be explained by a marked lowering of digestibility, and that, in fact, hydrolysis of the heated material restores its original nutritive value. These and similar studies of the effect of heating and cooking on the utilization of protein point the way to further investigation of this interesting subject.

6. THE DETERMINATION OF THE NUTRITIVE VALUE OF PROTEIN

In the determination of the dietary quality of proteins or of protein mixtures, mere elementary analysis, as for example, for nitrogen by the Kjeldahl method, affords little information concerning nutritive value. Two proteins, yielding practically identical elementary analyses, may by no means be utilized physiologically to the same extent. On the other hand, nitrogen values for protein or protein-like material, such as have been determined for a very large number of commonly employed food articles, are, of course, always useful in providing information as to the maximum and minimum limits for the content of protein, although nothing is

thus revealed concerning its nutritional quality. A more rational method of approach would appear to be to analyze the protein or protein-containing food quantitatively for all of the amino acids which are present and then to compare the composition on this basis with the requirements of the animal. This method has been useful for qualitative purposes but has not, as yet, come into use as a reliable quantitative method because first, the methods for the estimation of all of the amino acids are still laborious and largely inadequate from a quantitative standpoint, and second, the amino acid requirements as to kind and amount were not fully known. The second of these difficulties appears to be nearing a solution and if the first can be solved satisfactorily, it should then be possible to make a prediction as to the nutritive quality of a protein, provided that the particular protein is satisfactorily digested in the alimentary tract.

Lack of chemical methods for accomplishing the purpose has necessitated the employment of biological assay. The procedures are of two general types and are similar to those used in the study of individual amino acid requirements. One involves the ability of a protein, when incorporated in an otherwise adequate diet, to support growth or to maintain body weight. The other involves a measure of the nitrogen balance under certain specified conditions. The efficiency with which a protein is digested and made available for absorption, as well as the efficiency of utilization of absorbed amino acids following absorption, enters into the appraisal of a protein, when the growth criterion is employed, and the results are calculated in terms of grams of body weight increment per gram of protein ingested in the diet. The factor of digestibility is very often treated separately in calculating results from experiments on nitrogen balance. That is, the latter type of result is now generally expressed as that per cent of absorbed nitrogen which is retained in the body. It is clear, of course, that in the broad sense of nutritive value, digestibility is a factor which must be taken into account as may be seen in the extreme case of keratin. Keratin in the form of hair is refractory to digestion and therefore is incapable of furnishing any appreciable amount of useful nitrogenous compounds to the body. Both methods, the one depending on growth and the other on nitrogen balance, have vielded valuable information⁶ relative to a considerable number of pure proteins as well as

⁶ For extensive discussions of methods employed and results obtained, the reader is referred to Mitchell (47), Fixen (48), and Mitchell and Hamilton (40).

a great many different articles of food that are important as sources of protein to man and the domesticated animals. The proteins occurring in animal tissues (eggs, meat, and milk) are, as a class, superior in nutritive value to those present in foods of plant origin (cereal grains and legumes).

7. CHARACTERISTICS OF AMINO ACID DEFICIENCIES

A sufficiently pronounced lack of an indispensable amino acid in the diet leads to loss of body weight. This loss, which at first is rapid, continues until the animal dies. If a suitable supplement of the missing amino acid is introduced into the diet, the animal just maintains its body weight, whereas a larger supplement stimulates growth. Thus it appears that the difference between the requirement for maintenance of body weight and the requirement for growth is not a qualitative but rather a quantitative one. The fact that certain proteins or protein mixtures partly or entirely prevent loss in body weight without permitting growth, or that they appreciably spare loss of nitrogen from the body without permitting nitrogenous equilibrium or a positive nitrogen balance, is presumably explainable on this basis. In other words, a sharp cleavage between the demands for maintenance and growth in the sense that one amino acid might be required for maintenance and another for growth has not thus far been demonstrated. In this connection, however, it should be recalled that the animal organism is not able to synthesize arginine rapidly enough to provide for maximum growth.

Another aspect of amino acid deficiency is the decrease in food consumption. This is a phenomenon quite commonly observed with practically all types of dietary deficiencies. Seemingly, an animal confronted with a dietary shortage of an essential protein unit, automatically limits its total intake of food to correspond metabolically with the restricted supply of the one indispensable factor. Thus, although such an animal has access to unlimited quantities of the diet containing some of the limited substance, it may just manage to maintain body weight. If a few extra milligrams of the limiting amino acid are added to the diet each day, growth is initiated and the food consumption is increased. In such cases, the close relationship between increase in body weight and food consumption is best explained on the basis that the extra intake of the amino acid permits the physiological reactions of the cells to proceed at an accelerated rate and that the resulting increased demand for food is then reflected in the appetite.

The inhibition of growth in an animal by severe restriction of the intake of a single amino acid seems to result in a picture of partial starvation. The animals often exhibit abnormal coats and other general symptoms of a debilitated state such as are also seen in other types of deficiencies. Confirmed demonstrations of irreparable tissue change and sudden nutritive disaster such as characterize certain of the vitamin deficiencies are notably lacking.7 In fact, animals, stunted by the withholding of an indispensable amino acid for long periods, during which weight remains approximately constant, begin to grow at a normal or even accelerated rate when the dietary restriction is removed. One indispensable amino acid, tryptophane, is apparently not stored in the body in an available form for any considerable period of time as is shown by the fact that a given daily supplement of tryptophane supports better growth when given in two doses at intervals rather than in a single amount (49). The same situation may hold for other essential amino acids.

8. ARE THE DEMANDS FOR INDISPENSABLE AMINO ACIDS ABSOLUTE?

In the preceding discussion, the words "essential" and "indispensable" have been used with the meaning that certain amino acids must continually be present in the diet. However, it has been found that certain closely related derivatives of synthetic origin may, when supplied in the diet, suffice in place of the amino acid itself. In the case of tryptophane, for example, the corresponding indole lactic and indole pyruvic acids are utilizable in lieu of tryptophane. Thus, it is plain that, in this case, the dietary demand is not for the α -amino acid itself, but for a particular ring system and side chain. If phenyl pyruvic acid were demonstrated to take the place of phenylalanine,8 then the apparent specific demand would be not for any particular type of nitrogenous compound, but rather for a particular combination of the elements, carbon, hydrogen, and oxygen. Of course, in such a situation, the animal doubtless converts some of the lactic or pyruvic acid to the corresponding amino acid. Finally, since the corresponding lactic and pyruvic acids and similar utilizable derivatives do not occur naturally in diets, one is justified in referring to certain of the amino acids as essential dietary factors.

⁷ Rose (14a) has reported that animals deprived of valine exhibit unusual symptoms including sensitiveness to touch and lack of muscular coordination.

⁸ This has now been demonstrated by Rose (42a).

9. METABOLIC RELATIONSHIPS; SOURCE OF DISPENSABLE AMINO ACIDS

Investigation has yielded but little evidence of specific metabolic connections between the amino acids. Norleucine does not replace lysine (50); arginine, histidine; tyrosine, phenylalanine; nor cystine methionine. There is, nevertheless, as has been discussed, a distinct relationship between cystine and methionine. Homocystine (demethylated methionine) supplements a diet, which is low in the sulfur-containing amino acids, in supporting growth (51). Further, the possible origin of tyrosine in phenylalanine is among other conceivable and rather direct relationships of amino acids in the body.

Notwithstanding the fact that non-essential amino acids are not required in the diet, they are nevertheless ultimately present in animal proteins. A synthesis of these amino acids in the body requires some sort of starting material. Glycine apparently can be synthesized from nitrogenous compounds which otherwise would be excreted as urea. However, substances such as urea and ammonium salts have not been demonstrated to be utilizable by higher organisms unless through the intervention of microorganisms. It therefore appears that the dispensable amino acids must be synthesized from other protein units. 10 Curiously, evidence for this, in the case of glycine, could not be secured in tests with a limited number of other amino acids (27). If amino acids of some kind are necessary for the synthesis in the body of the amino acids not required in the diet, then the minimum possible intake of amino acids may be governed, not only by the minimal required amounts of all of the indispensable amino acids as determined singly in otherwise adequate diets, but also by a supply of amino acids from which the amino acids not required in the diet may be manufactured. Nevertheless, it seems entirely likely that the protein minimum of an animal, as determined in the optimal combination of amino acids, will prove to be lower than that secured with a protein like casein, which, despite being quite satisfactory in comparison with other proteins, does not, at restricted levels, supply the necessary amino acids in ideal proportions.

⁹ It is of interest to note that the loss of carbon dioxide from the ω -carboxyl group of β -hydroxyglutamic acid would yield one configuration of the essential amino acid, threonine.

¹⁰ An item of interest and possibly of significance is the fact that most of the indispensable amino acids fail to yield glucose, whereas nearly all of those now thought to be non-essential do yield glucose in the diabetic organism.

10. AMINO ACID REQUIREMENTS OF DIFFERENT SPECIES

It must be emphasized that most of the data available as to the amino acid requirements of the animal organism have been secured with a relatively few species and in particular with the rat as the experimental animal. Though it seems entirely possible or even probable and it has frequently been tentatively assumed that requirements of the higher organisms are the same in this regard. proof of this generalization remains to be secured. Evidence of limited value is had in the similar biological values found for the same protein tested with different species, and thus far, no species difference in the requirement for an indispensable amino acid, such as has been found, for example, with respect to vitamin C, has been disclosed. The determination of the kinds and the amounts of pure amino acids needed by the human being may prove of importance in medical treatment. As Rose has pointed out (42): "Indeed, it is not wholly improbable that methods may be devised whereby this ideal mixture may be employed for parenteral administration to human subjects when the prevention of undue loss of body structures is an important consideration."

11. SPECIFIC PHYSIOLOGICAL USES OF AMINO ACIDS

Protein either directly or following conversion to carbohydrate or fat can furnish energy to the body, but inasmuch as energy needs can be cared for adequately by preformed carbohydrate and fat, the chief interest in proteins and amino acids centers in other more specific functions. A growing animal obviously requires a constantly new supply of amino acids for use as structural units in the building of new cells, but the nature of the constant so-called "wear and tear" mechanism resulting in a continual demand for amino acids in the adult organism which needs only to preserve a kind of "status quo" with respect to body weight and nitrogen content still defies explanation. Is this latter demand for amino acids in part to replace protein molecules of cell tissue undergoing destruction? If so, is the destruction of a single molecule complete or only partial? On the other hand, is the demand for amino acids in the case of maintenance largely for more special purposes? There is little or no question that in the body, creatine, creatinine, purines (including uric acid), the many protein or protein-like enzymes and hormones, glutathione, taurine, etc., may have their ultimate origin in amino acids. And, though it has not been proved, histamine is probably synthesized from histidine; and epinephrine and thyroxine from tyrosine (see Chapter V). The unraveling of these and similar problems awaits future investigation. As Thomas (52), in referring to the biological values of proteins, has stated:

"What we need to know is: 1. Which amino acids must be present in food, 2. How much we require of each, 3. And to what purpose."

The recent rapid advances in discovering which amino acids are essential dietary constituents and in determining how much of each amino acid is required should now make possible further valuable study of "to what purpose" amino acids are utilized in the organism.

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APPENDIX

Table of pH, H⁺, and OH⁻ Values Corresponding to Electromotive Forces Determined in Hydrogen Electrode Measurements

By CARL L. A. SCHMIDT (Division of Biochemistry, University of California Medical School, Berkeley)

1. DISCUSSION

There are hardly any investigations relating to the physicochemical behavior of amino acids and proteins which do not require a knowledge of the pH of solutions of these compounds. The pH is quite commonly determined by means of the hydrogen electrode. For some purposes it may, at times, be more convenient to use the quinhydrone (1) or the glass electrode (2) to determine the pH.

Table II, which appears on page 1000, has been included in order to facilitate the conversion of the observed electromotive force values in hydrogen electrode measurements to pH, C_{H^+} , or C_{OH^-} . In calculations of this type the question of the value which is to be assigned to the calomel reference cell arises. It is needless to mention here the various questions which have arisen. They are discussed at length by Clark (3). In the present calculations the assumption has been made that the value of the "Tenth Normal Calomel Half-Cell," ($\|KCl\|(sat)\|KCl\|(0.1\|N)\|HgCl\|Hg$), at 25° is 0.336 volt, and that the value of a similar cell in which the N/10 KCl is replaced by N/1 KCl is 0.283 volt. The dissociation constant of water at 25°, K_w , is taken to be 1.012×10^{-14} .

Clark (1) gives 0.3376 volt as the value of the "Tenth Normal Calomel Half-Cell" at 25°, and 0.2458 volt for a similar cell in which the N/10 KCl is replaced by saturated KCl. The value of the

¹ To be more exact, it is the activity of the hydrogen ion rather than the concentration which is determined in hydrogen electrode measurements. In order to convert activity to concentration, it is necessary to know the value of the activity coefficient of the hydrogen ion. In many of the measurements involving biological material the value of the activity coefficient is not known. The assumption is usually made that the value of the activity coefficient is unity, which makes the numerical value of the hydrogen ion activity equal to that of the concentration.

N/1 KCl calomel electrode is 0.2852 volt. For the half cell, (\parallel KCl (0.1 N) \mid KCl (1.0 N), HgCl \mid Hg), the value 0.2848 volt at 25° is given. Values for these and other cells at a number of temperatures are given in Table I.

Whichever value is assigned to the calomel half-cell the conversion table can still be used. For example, if 0.338 volt is taken as the value for the "Tenth Normal Half-Cell," it is necessary to shift the values under $E_{\rm N/10}$ upward by two millivolts so that 0.338 volt will be opposite pH = 0 in order to obtain the pH value corresponding to the observed E.M.F.

In most biological work it is assumed that, by use of a saturated KCl-bridge, the contact potential is reduced to zero. This assumption may not be wholly valid. This assumption, together with the

Table I

Values for Half-Cells at Different Temperatures

			Hal	lf-Cell		
t	(1)	(2)	(3)	(4)	(5)	(6)
Degrees	Volt	Volt	Volt	Volt	Volt	Volt
18	0.3380	0.251	-0.229	-0.2668	0.7044	0.6423
20	0.3379	0.250	-0.231	-0.2686	0.7029	0.6404
25	0.3376	0.2458	-0.235	-0.2732	0.6992	0.6356
30	0.3371	0.242	-0.239		0.6955	0.6308
35	0.3365	0.238			0.6918	0.6261
38	0.3361	0.236			0.6896	0.6232
40	0.3358	0.234			0.6881	0.6213

Half-Cells:

- (1) KCl (sat.) KCl (0.1 N), HgCl Hg
- (2) KCl (sat.), HgCl | Hg
- (3) KCl (sat.) K H Phthalate (0.05 M) H₂ (1 atmos.), Pt.
- (4) KCl (sat.) 0.1 M Acetic Acid H₂ (1 atmos.), Pt.
- (5) $\|(H^+) = 1$, quinhydrone | Pt.
- (6) KCl (sat.) HCl (0.1 N), quinhydrone Pt.

(Clark, W. M., The Determination of Hydrogen Ions, Baltimore, 1928, p. 672.)

uncertainty regarding the value of the calomel half-cell, makes the estimating of pH somewhat arbitrary. However, in most biological work the accuracy with which the pH needs to be estimated is within the limits in which the uncertainties fall. In precision

999

measurements of pH it would be well for workers to report the value of the calomel half-cell used in the calculations.

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TABLE II

(The value of the "Tenth Normal Calomel Half-Cell" has been taken as 0.336 volt, and that of the "Normal Calomel Half-Cell" Table of pH, H⁺, and OH⁻ Values Corresponding to Electromotive Forces Determined in Hydrogen Electrode Measurements

	Сон_ ×10-ш ОН-	7.55 8.16	8.88 9.55	Con-	×10-11 OH-	1.128	1.21	1.41	1.53	1.78	1.93 08	2.25	2.43 2.63	2.84	3.08 3.08	3.59	3.88	4.7 54 54	4.91	5.30 7.9	6.21	6.70	2.52	8.43	9.13	9.90	COH-	1.07	T:-	1.10	1.25
	CH [↑] X10 ⁻² H ⁺	0.134 0.124	0.114	С _{н+}	×10 % H+	0.906	0.838	0.717	0.663	0.568	0.525	0.450	$0.416 \\ 0.385$	0.356	0.329	0.282	0.261	0.241	0.206	0.191	0.163	0.151	0.129	0.120	0.111	0.102	CHT - 10-12	0.947	0.911	0.870	0.811
	Hd	2.874	2.942	Ha		3.043	3.077	3.144	3.178	3.246	3.280	3.347	3.381 3.415	3.449	3.483	3.550	3.584	3.652	3.685	3.719	3.787	3.821	388	3.922	3 956	0.00	Ηď	4.023	4.040	4.024	4.091
	En/10	0.506	$0.510 \\ 0.512$	Ew/10		0.516	0.518	0.522	0.524	0.528	0.530	0.534	0.536	0.540	0.542	0.546	0.548	0.550	0.554	0.556	0.560	0.562	0.566	0.568	0.570	0.0	EN/10	0.574	0.575	0.577	$0.578 \\ 0.579$
le I.)	Емл	0.453 0.455	0.457	Ew.	107	0.463	0.465	0.469	0.471	0.475	0.477	0.481	0.483	0.487	0.489	0.493	0.495	0.497	0.501	0.503	0.507	0.509	0.513	0.515	0.517	0.015	EN/1	0.521	0.522	0.524	0.525 0.526
other values see discussion and Table I.)	Сон-	2.88	3.36		4.58	4.96 5.35	2.78	6.25	7.33	8.58	9.28	10.00	Con-	HO = OIX	1.08	1.26	1.37	1.60	1.72	1.86 02 03	2.18	25.35	2.75	2.98	2.21	3.76	4.06	4.75	5.14	5.99	6.49
ee discuss	С _Н + ×10-1 H+	0.352	0.301	0.258	0.221	0.204	0.175	0.162	0.138	0.128	0.109	0.101	CH+	X10-2 H.	0.886	0.801	0.741	0.034	0.587	0.543	0.465	0.430	0.368	0.340	0.315	0.269	0.249	0.213	0.197	0.169	0.156
values s	Ηď	1.454	1.521	1.589	1.657	1.691	1.758	1.792	1.860	1.893	1.961	1.990	Ηď	(2.023	2.096	2.130	2.10	2.232	2.265	2.333	2.367	2.434	2.468	2.502	2.570	2.603	2.671	2.705	2.772	2.840
or other	EN/10	0.422	0.426	0.430	0.434	0.436	0.440	0.442	0.446	0.448	0.452	U.454	EN/10		0.458	0.460	0.462	0.466	0.468	0.470	0.474	0.476	0.480	0.482	0.484	0.488	0.490	0.494	0.496	0.500	$0.502 \\ 0.504$
as 0.283 volt. For	E _{N/1}	0.369	0.373	0.377	0.381	0.383	0.387	0.389	0.393	0.395	0.399	0.401	EN/1		0.403	0.407	0.409	0.413	0.415	0.417	0.421	0.423	0.427	0.429	0.431	0.435	0.437	0.441	0.443	0.447	0.449
as 0.28	Con- X10-14 OH-	1.01	1.18	1.38	1.61	1.89	2.04	2.38	2.58	3.01	3.25	3.80	4.44	4.80	5.19	90.9	6.57	7.67	8.30	8.96 0.64	H 5	COH	1.05	1.13	1.22	1.43	1.54	1.80	1.95	2.28	2.46 2.66
	XNH+	1.000 0.925	0.856	0.732	0.627	0.536	0.496	0.425	0.393	0.336	0.311	0.266	$0.246 \\ 0.228$	0.211	0.195	0.167	0.154	0.145	0,122	0.113		CH+ CH+ CH+ CH+ CH+ CH+ CH+ CH+ CH+ CH+	0.968	0.895	0.828	0.709	0.656	0.561	0.519	0.444	0.411 0.380
	μđ	0.034	0.068	0.135	0.203	0.270	0.304	0.372	0.406	0.473	0.507	0.575	0.609	0.676	0.710	0.778	0.811	0.040	0.913	0.947	2	Ηď	.014								$\frac{1.386}{1.420}$
	E _{N/10}	0.336	0.340 0.342	0.344	0.348	0.352	0.354	0.358	0.360	0.364	0.368	0.370	0.372	0.376	0.378	0.382	0.384	3800	0.390	0.392	# 50.0	EN/10	0.396	0.398	0.400	0.404	0.406	0.410	0.412	$0.414 \\ 0.416$	$0.418 \\ 0.420$
	EN/1	0.283	0.287	0.291 0.293	0.295	0.299	0.301	0.305	0.307	0.311	0.313	0.317	$0.319 \\ 0.321$	0.323	0.325	0.329	0.331	0.000	0.337	0.339	1	EN/1	0.343	0.345	0.347	0.351	0.353	0.357	0.359	0.363	0.365 0.367

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Table II (Continued)

Сон- ×10-6 ОН-	2.56	2.66	25.79	200	3.10	3.22	33.00	3.49	3.63	000	00 V	4.23	4.40	4.58	4.75	4.40 1.40	0.14 9r	0 m	2.00	6.02	6.25	6.49	6.75	7.03	7 61	7.91	8.23	8.50	06.00	9.55	10.00	7	COH.	104	1.08	1.12	1.17	1.21	1.31	1.36	1.42	1.53	1.59	1 66
С _Н ⁺ ×10 ⁻⁸ H ⁺	0.396	0.381	0.367	0.555	0.326	0.314	0.305	0.290	0.279	0.700	070	0.239	0.230	0.221	0.213	0.204	0.197	0.100	0.175	0.168	0.162	_	0.150	0.144	0.153	0.128	0.123	0.119	0.114	0.106	0.101	(世25	0 975	0.938	0.902	0.868	0.835	0.772	0.743	0.714	0.661	0.636	0.611
Ηď					8.487	8.504	8.521	8.537	8.554	0.07	900	8.622	8.639	8.656	8.673	2.090	0.700	0.170	8 757	8 774	8.791	8.808	8.825	8.842	8.839	8.892	8.909	8.926	0000	8.977	8.994	, t	hц	0 011	9.028	9.045	9.062	9.078	9.112	9.129	9.146	9.180	9.197	0 914
EN/10	0.833	0.834	0.835	0.850	888	0.839	0.840	0.841	0.842	0.843	1000	0.846	0.847	0.848	0.849	0.850	0.851	0.00	0.854	855	0.856	0.857	0.858	0.859	0.800	0.862	0.863	0.864	0.00	0.867	898.0	ţ	EN/10	0 869	0.870	0.871	0.872	0.873	0.875	0.876	0.877	0.879	0.880	0.881
EN/1	0.780	0.781	0.782	0.783	0.785	0.786	0.787	0.788	0.789	0.790	0.700	0.793	0.794	0.795	0.796	767.0	0.798	882.0	801	0.802	0.803	0.804	0.805	0.800	208.0	0.809	0.810	0.811	0.012	0.814	0.815	ļ	EN/1	0.816	0.817	0.818	0.819	0.820	0.822	0.823	0.824	0.826	0.827	868 0
Сон-	3.79	3.94	4.10	4.25 4.44	4 60	4.80	4.99	5.19	38	0.00	•	62.9	6.53		7.08	7.03	7.07	16.0	0 ×	90	9.58	9.64	10.02	•	×10-6 OH-	1.04	1.08	1.13	1.17	1.27	1.32	1.37	1.43	1.40	1.60	1.66	1.73	1.80	1.05	2.02	2.10	2.27	2.36	9 46
С _В + ×10-7 н+		0.257	0.247	0.738	0.550	0.211		0.195	0.188	181.0	0.174	0.101	0.155	0.149	0.143	0.138	0.132	0.127	0.120	113	0.109	0.105	0.101	-1	十二 10 mm 10 mm 10 mm	0.970	0.933	0.897	0.809	0.000	0.768	0.739	0.710	0.039	0.0	0.608	0.585	0.562	0.520	0.200	0.481	0.445	0.428	0.419
Ηď		7.591	7.608	7.624	7 658	7.675	7.692	7.709	7.726	7.743	7.700	7 794	7.810	7.827	7.844	7.801	8/8.7	7.030	7 999	7 946	7.963	7.980	7.996		Пď		8.030	8.047	5.00	8008	8.115	8,132	8.149	8.100	8 199	8.216	8.233	8.250	8.284	8.301	8.318	8.351	8.368	388
EN/10	0.784	0.785	0.786	0.787	789	0.790	0.791	0.792	0.793	0.794	0.795	0.797	0.798	0.799	0.800	0.801	0.802	0.803	908	808	0.807	0.808	0.809	ŗ	EN/10	0.810	0.811	0.812	0.819	0.014	0.816	0.817	0.818	0.019	0.821	0.822	0.823	0.824	0.826	0.827	0.828	0.830	0.831	0 835
E _N /1	0.731	0.732	0.733	0.734	0.736	0.737	0.738	0.739	0.740	0.741	247	0.744	0.745	0.746	0.747	0.748	0.749	0.750	0.751	0.100	0.754	0.755	0.756	ŗ	EN/1	0.757	0.758	0.759	0.760	0.762	0.763	0.764	0.765	0.700	0.768	0.769	0.770	0.771	0.773	0.774	0.775	0.777	0.778	0 220
Con-	6.10	6.32	6.57	48.5	7.30	7.67	7.97	8.30	8.65	96.80	9.97	0.0		C _{OH} -	X10-1 OH-	1.05	1.09	1.13	1.18	1.65	33.	1.38	1.43	1.49	1.55	1.01	1.74	1.81	88.	2.30	2.12	2.20	2.58	2.38	27.47	2.67	2.78	2.89	30.00	3.24	3.37	3.65		
CH+ V10-6 H+	0.166	0.160	0.154	0.148	0.142	0.132	0.127	0.122	0.117	0.113	0.108	100		С _Н +		0.964	0.927	2832	200	0.020	0.763	0.734	0.706	0.679	0.653	0.020	0.581	0.559	2538	0.017	0.478	0.460	0,443	0.420	0.304	0.379	0.364	0.350	0.657	0.312	0.300	0.277		
Ηď		6.796		6.830	6.847	6.881	6.898	6.914	6.931	6.948	0.000	0000		Hď		7.016	7.033	000.	7.067	7 100	7.117	7.134	7.151	7.168	7.185	7 219	7.236	7.253	7.269	7.250	7.320	7.337	7.354	7.371	7.405	7.422	7.439	7.455	7.472	7.506	7.523	7.557		
EN/110	0.737	0.738	0.739	0.740	0.741	0.743	0.744	0.745	0.746	0.747	0.748	0.750		EN/10	;	0.751	0.752	0.753	0.754	0.756	0.757	0.758	0.759	0.760	0.761	0.763	0.764	0.765	0.766	768	0.769	0,770	0.771	0.772	0.774	0.775	0.776	0.777	0.770	0.780	0.781	0.783		* Nontrol noint
EN/1	0.684	0.685	0.686	0.687	0.000	0.690	0.691	0.692	0.693	0.694	0.090	*0.090		EN/1	000	0.698	0.699	200	707	703	707	0.705	0.706	0.707	208	0.710	0.711	0.712	0.713	0.714	0.716	0.717	0.718	0.719	0.721	0.722	0.723	0.724	0.726	0.727	0.728	0.730	1	* Non+

COHT X10-3 OH-		4.00	4.32	7.69 5.06	5.47	5.92	88	7.44	80.03	9.72		Con		1.10	1.19	1.29	1.50	1.62	1.76	2.02	2.22	2.40 0.40	2.80	3.03	2.6	3.83	4.13	4.48	5.22	5.65	6.10	7.13	7.73	8.36	9.73
X10-11 H	0.295	0.273	0.234	0 216	0.185	0.171	0.109	0.136	0.126	0.116	5	C田+ C田+	. Нат-OIX	0.93	0.850	0.786	0.728	0.623	0.576	0.555	0.456	0.422	0.361	0.334	0.308	0.264	0.245	0.220	0.203	0.179	0.166	0.153	0.131	0.121	0.104
рн 11 408	11.530	11.563	11.631	11.665	11.732	11.766	11.800	11.868	11.901	11.935		μd		12.009	12.071	12,104	12.138	12.206	12.240	12.2/3	12.341	12.375	12.443	12.476	12.510	12.578	12.612	12.645	19.713	12.747	12.781	12.814	12.882	12.916	12.983
EN/18	1.018	1.020	1.024	1.026	1.030	1.032	1.034	1.038	1.040	1.042	1.044	EN/19	970	1.040	1.050	1.052	1.054	1.058	1.060	1.002	1.066	1.068	1.070	1.074	1.076	1.080	1,082	1.084	1.080	1.090	1.092	1.094	1.080	1.100	1.102
EN/1	0.965	0.967	0.971	0.973	0.975	0.979	0.981	0.00	0.987	0.989	0.891	EN/1	000	0.993	0.997	0.999	1.001	1.005	1.007	1.009	1.013	1.015	1.017	1.021	1.023	1.025	1.029	1.031	1.033	1.037	1.039	1.041	1.046	1.047	1.049 1.051
X10-4 OH-	1.03	1.20	1.30	1.52	1.65	1.78	2.08	2.24	2.43	83.5	3.07	2.32 70 70	900	4.18	4.52	8.83 80.83	5.75	6.17	6.66 7.93	7.78	8.43	0.00		COH	X10-8 OH-	2.5	1.24	1.35	1.45	1.04	1.84	1.99	2.15	25.5	2.71
X10-10H+	0.981	0.840	0.777	0.665	0.615	0.569	0.520	0.451	0.417	0.000	0.330	0.305	0.202	0.242	0.224	0.207	0.177	0.164	0.152	0.130	0.120	0.111		CH+	X10-11 H	0.950	200	0.752	0.696	0.644	0.551	0.509	0.471	0.403	0.373
Hď		10.042	10.110	10.143	10.211	10.245	10.273	10.346	10.380	10.414	10.481	10.515	10.049	10.617	10.651	10.684	10.718	10.786	10.820	10.887	10.921	10.955		Hď		11.022	11.000	11.124	11,158	11.191	11 259	11.293	11.327	11.361	11.428
EN/10	0.928	0.830	0.934	0.936	0.940	0.942	0.044	0.948	0.950	0.952	0.956	0.958	0.960	0.962	0.966	0.968	0.970	0.974	0.976	0.80	0.982	0.984	0.300	EN/118		0.988	0.880	0.994	0.996	0.998	1.000	1.004	1.006	1.008	1.012
En/1	0.875	0.877	0.881	0.883	0.887	0.889	0.891	0.895	0.897	0.899	0.903	0.902	0.907	0.90	0.913	0.915	0.917	0.921	0.923	0.829	0.929	0.931	0.899	En./1		0.935	0.937	0.959	0.943	0.945	0.947	0.05	0.953	0.955	0.929
Con- V10-60H-	1.72	1.79	1.93	2.01	2.17	2.26	27.00	2.54	2.64	4.6	2.97	3.00	3.21	3.47	3.60	3.75	6.08 0.55	4.22	4.38	4.73	4.91	5.11	5.53	5.75	9.6	6.45	6.70	20.00	7.55	7.84	8.16	000	9.50	9.55	26.6
С _Н ⁺	0.588	0.566	0.523	0.503	0.466	0.448	0.431	0.398	0.383	0.369	0.341	0.328	0.315	0.50%	0.281	0.270	0.260	0.240	0.231	0.214	0.200	0.198	0.183	0.176	0.169	0.157	0.151	0.145	0.159	0.129	0.124	0.119	0.110	0.100	0.102
Hď	9.231	9.248	9.281	9.298	9.332	9.349	9.366	9.400	9.417	9.434	9.467	9.484	9.501	0.010	9.552	9.569	9.585	9.619	9.636	9.003	9.687	9.704	9.738	9.755	9.772	9.805	9.822	9.830	0.000	9.890	9.907	9.924	9.941	9.974	166.6
EN/10	0.882	0.883	0.885	0.886	0.888	0.889	0.830	0.892	0.893	0.894	0.890	0.897	0.898	666	0.901	0.902	0.903	0.30	0.906	7000	0.909	0.910	0.911	0.913	0.914	0.916	0.917	0.918	0.919	0.921	0.922	0.923	0.924	0.926	0.927
EN/1	0.829	0.830	0.832	0.833	0.835	0.836	0.837	0.000	0.840	0.841	0.042	0.844	0.845	0.840	0.848	0.849	0.820	0.851	0.853	0.854	0.856	0.857	828	0.860	0.861	0.863	0.864	0.865	0.800	0.868	0.869	0.820	0.872	0.873	0.874

Table of pH, H⁺, and OH⁻ Values Corresponding to Electromotive Forces Determined in Hydrogen Electrode Measurements

$\mathrm{E}_{\mathrm{N}/1}$	$\mathrm{E}_{\mathrm{N}/10}$	pH	C _H + ×10 ^{−13} H+	$^{\mathrm{C}_{\mathrm{OH}}}_{ imes 10^{-1} \mathrm{OH}}$
1.053	1.106	13.017	0.961	1.05
1.055	1.108	13.051	0.889	1.14
1.057	1.110	13.085	0.822	1.23
1.059	1.112	13.119	0.761	1.33
1.061	1.114	13.153	0.704	1.44
1.063	1.116	13.186	0.651	1.55
1.065	1.118	13.220	0.602	1.68
1.067	1.120	13.254	0.557	1.82
1.069	1.122	13.288	0.516	1.96
1.071	1.124	13.322	0.477	2.12
1.073	1.126	13.355	0.441	2.29
1.075	1.128	13.389	0.408	2.48
1.077	1.130	13.423	0.378	2.68
1.079	1.132	13.457	0.349	2.90
1.081	1.134	13.491	0.323	3.13
1.083	1.136	13.524	0.299	3.38
1.085	1.138	13.558	0.277	3.65
1.087	1.140	13.592	0.256	3.95
1.089	1.142	13.626	0.237	4.27
1.091	1.144	13.660	0.219	4.62
1.093	1.146	13.693	0.203	4.99
1.095	1.148	13.727	0.187	5.41
1.097	1.150	13.761	0.173	5.85
1.099	1.152	13.795	0.160	6.32
1.101	1.154	13.829	0.148	6.84
1.103	1.156	13.863	0.137	7.39
1.105	1.158	13.896	0.127	7.97
1.107	1.160	13.930	0.117	8.65
1.109	1.162	13.964	0.109	9.28
1.111	1.164	13.998	0.101	10.02
			×10 ⁻¹⁴ H ⁺	×N OH-
1.113	1.166	14.032	0.930	1.09

⁽Schmidt, C. L. A., and Hoagland, D. R., Univ. of Calif. Pub. Physiol., 5, 23 (1919).)

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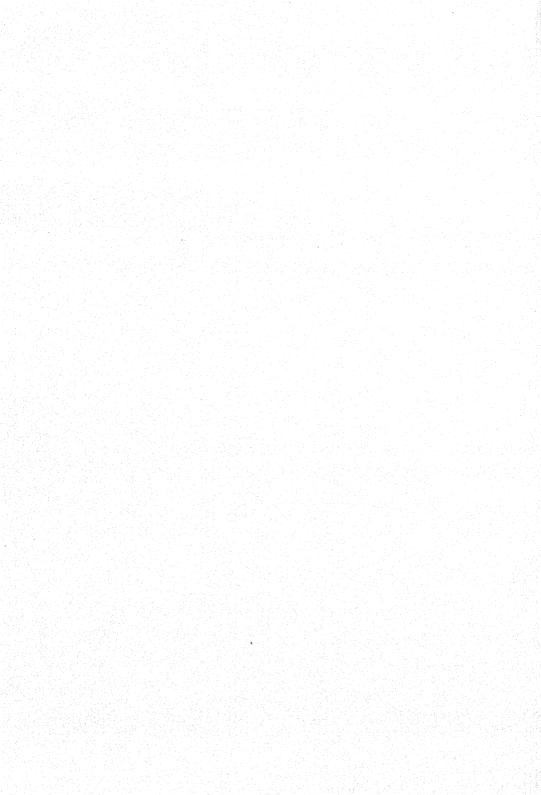
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ADDENDUM TO

THE CHEMISTRY

OF THE

AMINO ACIDS AND PROTEINS

INCLUSIVE OF SOME OF THE ADVANCES SINCE 1937

Edited by

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Der fortschrift der Wissenschaft ist heutzutage nicht so sehr durch geniale Leistungen einzelner Forscher, als vielmehr durch das planmässige Zusammenarbeiten vieler Beobachter bedingt.

EMIL FISCHER
Nobel lecture

CHAPTER II

THE CONSTITUTION AND SYNTHESIS OF THE AMINO ACIDS*

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In a recent discussion of the criteria for acceptance of an amino acid, Vickery (505) has stated that there are (a) eighteen amino acids concerning which there is no doubt whatever, (b) seven amino acids that occupy a special position because of their narrow range of distribution or other reasons, (c) five amino acids known as plant constituents that may possibly be expected to be found in proteins, and (d) twenty-two amino acids for which claims have not been substantiated. The following comment by Dakin (506) is of interest in this connection. "It seems certain, however, that the natural acid (named β -hydroxyglutamic acid (507) in 1918) cannot be a β -hydroxy acid. Failure to convert it into a ketoglutaric acid, or glutaconic acid derivatives, and its limited reaction with periodic acid, and other evidence all support this view."

1. Stereochemical Relations. It is now known that the naturally occurring amino acids belong to the l-series of configurationally related compounds (508). This significant generalization has been derived from the experiments of Fischer, Freudenberg, and other workers who found that the optical rotations of l(+)-lactic acid and the natural antipodes of alanine and other amino acids exhibit analogous changes and that many natural amino acids are interconvertible or may be converted into the same derivative. The rule of Lutz and Jirgensons (509) that the rotations of l-amino acids become more positive with increasing molecular equivalents of hydrochloric acid is a convenient criterion for the determination of the stereochemical relations of the amino acids.

Designation of natural amino acids as l(+) or l(-) and the unnatural antipodes as d(+) or d(-) signifies that the configuration is l or d and that the rotation of the free amino acid in water at

^{*} The valuable technical assistance of Mr. Edwin L. Sexton and Mr. John Murray is gratefully acknowledged.

25° is positive (+) or negative (-). This nomenclature has been adopted by many authors to make certain that the ambiguities, present all too frequently in the older literature, may be avoided. The symbols for the natural forms of the accepted amino acids are given below. Although natural threonine has the l-configuration of the groups on the α -carbon atom, it is designated as d(-) because it has been shown to be analogous to d(-)-threose in its spatial relations (510).

Table I
Symbols for the Natural Forms of the Accepted Amino Acids

Alanine	l(+)	Lysine	l(+)
Arginine	l(+)	Methionine	l(-)
Aspartic acid	l(+)	Norleucine	l(+)
Cysteine	l(-)	Phenylalanine	l(-)
Cystine	l(-)	Proline	l(-)
Diiodotyrosine	l(-)	Serine	l(-)
Glutamic acid	l(+)	Threonine	d(-)
Glycine	Inactive	Thyroxine	l(?)
Histidine	l(-)	Tryptophane	l(-)
Hydroxyproline	l(-)	Tyrosine	l(-)
Isoleucine	l(+)	Valine	l(+)
Leucine	l(-)		

2. Synthesis. Methods for the synthesis of amino acids which have been reported recently are described below.

The condensation of alkyl and aryl halides with the sodium derivative of ethyl benzamidomalonate has been applied by Redemann and Dunn (511) and Painter (512) to the synthesis of alanine, α -amino- γ -hydroxy-n-butyric acid lactone, α -amino- γ -phenoxy-n-butyric acid, aspartic acid, glutamic acid, glycine, leucine, nor-leucine, phenylalanine, and valine. The attempted synthesis of β - and γ -halogen amino acids by this method failed.

Small amounts of lysine, ornithine, and α - γ -diamino-n-butyric acid were synthesized by Adamson (513) who employed the Schmidt hydrazoic acid reaction (replacement of the distal carboxyl group in an α -aminodicarboxylic acid by an amino group).

The large-scale preparation of *lysine* by the Beckmann rearrangement of cyclohexanone oxime has been described by Eck and Marvel (514). A comparable synthesis of *ornithine* and *citrulline* from cyclopentanone oxime has been reported by Fox, *et al.*, (515) and Maeda and Nozoe (516).

A number of amino acids have been synthesized by amination of appropriate α -halogen acids. *Valine* (300 gm. lots in 42 per cent of the theoretical yield calculated from isovaleric acid) was pre-

pared from α-bromoisovaleric acid by Marvel (517). Alanine (38-40 gm. in 65-68 per cent of the theoretical yield) was prepared from α-bromopropionic acid by Tobie and Ayres (518). Serine (140 gm. lots in 30-40 per cent over-all yields calculated from methyl acrylate) was prepared from α -bromo- β -hydroxypropionic acid by Carter and West (519) essentially by the method of Schiltz and Carter (520). Glycine (220-230 gm. in 59-61 per cent of the theoretical yield) was prepared from chloroacetic acid by Cheronis and Spitzmueller (521). α -Amino-n-butyric acid (59-62 gm. in 57-60 per cent of the theoretical yield) was prepared from α-bromo-nbutyric acid by Cheronis and Spitzmueller (521). The factors concerned in the amination of α -halogen acids with ammonia and ammonium carbonate have been investigated by Cheronis and Spitzmueller (521), Chadwick and Pacsu (522), and Dunn, et al. (523). The effects of light, oxygen, and other factors on the bromination of aliphatic acids have been studied by Kharasch and Hobbs (524).

The Michael condensation (addition of sodium enol malonates to α , β -unsaturated acids) has been applied by Marvel and Stoddard (525) to the preparation of 225–250 gm. (70–75 per cent of the theoretical amount) of *glutamic acid* from methyl acrylate and diethyl phthalimidomalonate. Reactions of this type have been studied extensively in recent years by Connor, *et al.* (526).

The preparation of α -amino- β -hydroxy acids by the addition of methyl alcohol and mercuric acetate to acrylic acid derivatives was first used by Schrauth and Geller (527) for the synthesis of α -amino- β -hydroxyisovaleric acid. This procedure has been adapted by Abderhalden and Heyns (528) to the preparation of threonine; by Carter, et al. (529) to the dl- and optically active forms of threonine and allo-threonine; by Abderhalden (530) to β -hydroxyleucine and β -hydroxynorleucine; and by Carter, et al. (519, 520) to serine. Ethyl- α -bromo- β -ethoxypropionate, intermediate in the synthesis of serine, has been prepared by Wood and du Vigneaud (531) by the addition of bromine to ethyl acrylate and the action of sodium ethylate on the resulting ethyl- α , β -dibromopropionate. It has been recommended that the ethyl- α -bromo- β -ethoxypropionate be used in the crude state since difficulties are sometimes encountered in its distillation (532).

The preparation of α -amino acids by the reduction of α -oximino (isonitroso) acetoacetic esters was first applied by Bouveault and Locquin (533) to leucine and isoleucine. In 1909, Schmidt and

Widmann (534) synthesized alanine, norleucine, and α -amino-n-butyric acid ester hydrochlorides by this procedure. Analogous syntheses which have been reported include hydroxyglutamic acid from ethyl α -isonitrosoacetonedicarboxylate (535), glutamic acid from diethyl- α -oximinoglutarate (536), α -amino-n-butyric acid from ethyl α -oximinoacetoacetate (536), hydroxyprolines (a) and (b) from α -oximino- δ -chloro- γ -valerolactone (536, 537), and threonine and allo-threonine from ethyl O-ethyl oximinoacetoacetate (538). When α -oximinoacetoacetic ester is reduced, the product is α -amino-n-butyric acid (536) or a pyrazine (539); however, α -amino- β -hydroxy-n-butyric acid results when the O-ethyl derivative of this oximino ester is reduced and hydrolyzed (536).

In 1902, Fischer and Groh (540) synthesized alanine by the reduction of the phenylhydrazone of pyruvic acid. Comparable procedures have been employed by Feofilaktov, et al., in the preparation of phenylalanine (541), alanine (542), valine (543), leucine (544), isoleucine (544), and allo-isoleucine (544). The hydrazones of the α -keto acids required in the synthesis of these amino acids were prepared by the action of phenyldiazonium salts on alkyl acetoacetic esters.

The degradation of substituted malonic acid hydrazids, a method utilized by Curtius and Sieber (545) in the synthesis of alanine, has been applied by Darapsky, et al. (546) to the preparation of glycine, valine, leucine, and α -aminoisoamylacetic acid from substituted cyanoacetic esters. The same procedure has been employed by Gaudry and King (547) in the synthesis of β -phenylalanine. The amino acids α -aminoisobutyric acid, α -aminodiethylacetic acid and α -aminodiisopropylacetic acid have been synthesized by Kuo-Hao Lin, et al. (548) from dialkyl α -cyanoacetates through the intermediates, ethyl dialkylmalonamate and ethyl N-bromo dialkylmalonamate.

Racemization of l(+)-glutamic acid by heating the amino acid in the dry state (549, 550), in acid solution (551), or in basic solution (552) has been used to prepare large amounts of dl-glutamic acid. Benzyl-dl-cysteine, intermediate in the preparation of d(+)-cystine, was prepared by racemizing benzyl-l(-)-cysteine by means of acetic anhydride (531).

Quantities of l(-)-3,5-diiodotyrosine ranging from 155-168 gm. (66-72 per cent of the theoretical amount) were prepared by Savitskii (553) by the action of iodine (in aqueous ammonia) on tyrosine. Nagase (554) synthesized a small amount of 3,5-diiodo-

tyrosine by the action of iodine on anhydrohydroxydimercurityrosine (prepared from tyrosine and mercuric acetate). Monoiodo and diiodo derivatives of tyrosine were isolated by Ludwig and von Mutzenbecher (555) from the acid hydrolysate of casein which had been treated with sodium bicarbonate and iodine. A low yield of thyroxine was obtained by the action of iodine on alkaline solutions of casein and other proteins and by treatment of l(-)-diiodotyrosine with sodium hydroxide in the experiments of von Mutzenbecher, et al. (555–557), Harington and Rivers (558), and Block, Jr. (559).

A general method for the preparation of α -N-alkylamino acids has been devised by Cocker, et al. (560). N-benzenesulfonyl, N-m-xylene-4-sulfonyl, and N-mesitylenesulfonyl derivatives of glycine and alanine were converted to N-alkylamino derivatives by the action of alkyl halides and base. The aromatic sulfonyl groups were hydrolyzed with sulfuric acid and removed from the solution as insoluble zinc or magnesium sulfonate. The α -alkylamino acids were crystallized by conventional methods. Six α -alkylamino acids were prepared in satisfactory yields but the method gave low yields, or failed entirely, with N-isopropyl-, N-benzyl-, N-n-hexyl-, and N-n-heptylglycines.

Additional investigations to which only brief reference may be made because of the limitations of space include the following: Preparation of azlactones, conversion of allo-threonine to threonine, acetylation of amino acids in pyridine, and synthesis of α-aminoβ-thiol-n-butyric acids (561); attempted synthesis of phenylserine through the intermediate α -bromo- β -methoxy- β -phenylpropionic acid (562); synthesis of an analog of thyroxine (563), an isomer of thyroxine (564), and a homologue (ethionine) of methionine (565); synthesis of isocystine (566), isocysteine (566), and homocysteine (567); preparation of phenylalanine (568), alanine and dipeptides (569), and cyclohexylalanine (570) by the catalytic reduction and hydrolysis of the oximes of α -keto acids; synthesis of glutamic acid by the hydrolysis and hydrogenation of α -acetamino- α -hydroxyglutaric lactone (prepared by the condensation of α -ketoglutaric acid and acetamide) (571); preparation of citrulline by the acid hydrolysis of arginine (572); synthesis of homoarginine (from lysine) (573) and 6-methoxytryptophane (574); selective racemization of α -amino acids and dipeptides by ketene (575); preparation of amino acids containing the isotopic atoms deuterium and N^{15} (508); studies of the stability of hydrogen-carbon and deuteriumcarbon linkages in glutamic acid (576); asymmetric syntheses by enzyme systems of anilides of acylated peptides (577); isolation of a hydroxylysine from gelatin (578); synthesis of l(+)-citrulline from l(+)-ornithine or l(+)-arginine (579) essentially by the method of Kurtz (580); synthesis of β -hydroxyvaline (581) and β -hydroxynorvaline (582); isolation from wool treated with sodium carbonate (583) and synthesis (584) of lanthionine (β -amino- β -carboxyethyl sulfide); isolation of a selenium-containing amino acid from plant material (585); isolation (586, 587), spatial configuration (588), and properties (589–591) of canavanine; and the synthesis of aminocyclohexane carboxylic acids (592).

3. Origin of Amino Acids in Plants and Animals. The material in this section is limited to special topics referred to in review articles particularly those of Schoenheimer, et al. (593, 594) and Lewis and Garner (595).

It has been established by experiments with amino acids containing the isotopes H² (deuterium), N¹⁵, and S³⁵ (596), that amino acids and cell proteins of plants and animals undergo rapid and continuous change. It may be assumed, therefore, that the deamination and amination of amino acids, the opening and closing of peptide bonds, the partial degradation of proteins, and the synthesis of proteins are dynamic processes characteristic of living matter. In the normal animal there occurs a continuous transfer of nitrogen and chains of carbon atoms. The theoretical possibility that proteins may be interconverted by chemical reactions affecting only the free groups of amino acid residues without cleavage of peptide bonds has been discussed by Schmidt, et al. (597).

The intermolecular transfer of amino groups between α -amino acids and α -keto acids (called transamination) is a reversible reaction which reaches equilibrium rapidly. Reactions of this type are limited to the l-antipodes mainly of the amino acids alanine, aspartic acid, and glutamic acid and the α -keto derivatives of these amino acids. The transfer of amino groups is effected by the action of the enzyme, transaminase, which is widely distributed in microorganisms, tumors, and plant and animal tissues.

The conversion of certain amino acids into others has been demonstrated by metabolic studies. It has been shown that tyrosine is formed from phenylalanine; glutamic acid from α -ketoglutaric acid; α -ketoglutaric acid from pyruvic acid and carbon dioxide; arginine, proline, and glutamic acid from ornithine; arginine from ornithine and citrulline; d(+)-leucine from l(-)-leucine; and cystine from methionine.

The amino acid requirements of animals differ according to species and the physiological responses employed as criteria. Glycine is an essential growth factor for the chick but not the rat. Norleucine is essential for the maintenance of nitrogen equilibrium but not the growth of the rat. Of the ten amino acids found by Rose to be essential for the growth of the rat, only isoleucine, methionine, phenylalanine (or tyrosine), threonine, tryptophane, and valine appear to be essential for the maintenance of nitrogen equilibrium.

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- The following additional syntheses have been reported:
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CHAPTER III

SECTION I. THE ISOLATION OF THE AMINO ACIDS FROM PROTEINS

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- 1. Introduction. The isolation of large quantities of pure amino acids from proteins or amino acid mixtures has become an important link in the ever-increasing studies in nutrition. Pure amino acids are needed for the preparation of synthetic foods and in investigations on the relation of diet to detoxication. The extensive research on the chemistry of cancer has included attempts to isolate abnormal amino acids from the tumor tissue. A crystalline selenium-containing amino acid (131) has been isolated from plant material grown in seleniferous areas. It is a dicarboxylic acid with the proposed formula HOOC·CH(NH₂)·CH₂·Se·CH₂·CH₂·CH₄·CH(NH₂)·COOH. An additional sulfur-containing amino acid, lanthionine (132) has been isolated from wool treated with sodium carbonate before acid hydrolysis.
- 2. Hydrolysis and General Methods. The course of the hydrolysis of proteins can be followed by determining the degree of optical rotation of the non-protein products (133). The separation of the constituents of the protein hydrolysate by fractionation with an azeotropic solvent mixture (134) has been reported. The solvents, 91 per cent isopropyl alcohol, or 80 per cent dioxane, or 72 per cent n-propyl alcohol, were used to treat the concentrate of a strong acid hydrolysate giving arginine and histidine in the aqueous phase and the important monoaminomonocarboxylic acids in the nonaqueous phase. Synge (135) has made use of the differences in the partition coefficients in chloroform-water and ethyl acetate-water systems of the N-acetyl derivatives of the amino acids. These partition coefficients vary sufficiently to permit separation of many of the amino acids in good yields. The hydroxyamino acid fraction can be isolated by making use of the N-acetyl-O-benzoyl derivatives, and the individual hydroxyamino acids can be separated by

making use of the variation in the partition coefficients in chloroform-water of the N-acetyl-O-methyl derivatives.

- 3. Isolation of Individual Amino Acids. A number of isolation procedures for individual amino acids has been reported. Lysine has been isolated after benzoylation of the copper salts of the amino acids of a protein hydrolysate (136). The insoluble lysine complex precipitate and l(+)-lysine dihydrochloride are obtained in 20-60 per cent overall yield by decomposition of the copper complex and subsequent hydrolysis to remove the benzoyl group. Lysine has also been obtained as the picrate from an acid (H₂SO₄) protein hydrolysate made neutral with barium or calcium hydroxide (137). Fractional crystallization of the picrate yields pure lysine and also histidine as a by-product. Gilson (138) has simplified the Hanke and Koessler method for the preparation of histidine by precipitation with mercuric chloride. l(-)-serine has been isolated from silk fibroin by precipitating it with p-hydroxyazobenzene-p'-sulfonic acid in an acid-free hydrolysate from which tyrosine, glycine, and alanine had been removed (139). The salt was decomposed with barium acetate giving l(-)-serine in a 75 per cent overall yield. l(-)-Phenylalanine has been isolated, but not in very good yield, by preparing the picrolonate of the fraction salted out of a protein hydrolysate at pH 6, fractionally crystallizing this derivative, and liberating the free amino acid by subsequent hydrolysis (140). d(+)-Cystine has been prepared by the racemization of S-benzyl-l-cysteine with acetic anhydride, conversion into the N-formyl-S-benzyl-dl-cysteine, resolution with brucine, subsequent conversion to d(+)-cysteine, and then oxidation to d(+)-cystine (141). l(-)-Cystine, the starting material, was reduced to l(-)-cysteine with liquid ammonia and sodium.
- 4. Racemization of Amino Acids. Racemization of amino acids has been accomplished by the use of ketene (142, 143). Acetylation with ketene will give the optically active or the racemic N-acetyl derivatives depending upon the condition of acidity. l(+)-Glutamic acid can be racemized by heating at 190–195° for several hours, giving dl-pyrrolidone carboxylic acid which, upon hydrolysis with sodium hydroxide, gives dl-glutamic acid in 70 per cent yield (144).
- 5. Resolution of Amino Acids. A fourth method for the resolution of amino acids (see page 161) is by the use of enzymes. Asymmetric enzymic synthesis has been used to resolve *dl*-phenylalanine (145).

Using acetyl-dl-phenylalanyl-glycine and aniline as starting materials, and cysteine-papain as the enzyme system, it was found that the l-form combines with aniline more rapidly than does the d-form. d(-)-Glutamic acid has also been prepared by the enzymic resolution of carbobenzoxy-l-glutamic acid with papaincysteine and aniline (146). Carbobenzoxy-l-glutamic acid-anilide is formed leaving behind the carbobenzoxy-d-glutamic acid which, on hydrogenation and conversion to the hydrochloride, yields pure

TABLE I

Specific Retardation Volumes in cc per Gram Adsorbent for 0.5 per cent
Solutions of Various Amino Acids and Peptides

Substance	Medium	Adsorbent	Spec. ret vol.
Glycine	0.1 M NaCl	Carbo Active	0
Alanine	«	«	0.3
Valine	u	α	3.2
Leucine	"	a	7.7
Isoleucine	u	ű	9.2
Proline	a .	"	2.5
Hydroxyproline	u	u	1.9
Asparagine	0.05 M Na ₂ SO ₄	"	2.0
Betaine-HCl	"	u .	2.3
Phenylalanine	0.1 M NaCl	"	62.5
Tryptophane	"	"	76.5
Hippuric acid (0.29%)	0.1 M Na ₂ SO ₄	"	122
Histidine-HCl	0.1 M NaCl	"	15.1
Aspartic acid (sat. sol.)	Glycine buff. pH 2.96	"	3.6
Glutamic acid	"	"	5.5
Glycylglycine	0.1 M NaCl	"	3.5
Leucylglycine	"	"	18.2
Leucylglycylglycine	"	"	29.8
Ornithine	Glycine buff. pH 9.92	Eponit 3n	2.7
Lysine		"	9.7
Histidine	(u	15.4
Arginine	u	"	23.6

(Tiselius, A., Ark. Kemi, Mineral., Geol., 15 B, 1 (1941)).

d(-)-glutamic acid after a few recrystallizations. Another general enzymic method is the use of d-amino acid oxidase obtained from swine kidney (147). In the presence of lithium hydroxide at pH 8.3 to 8.5, the d-form is oxidized so that dl-alanine, for example, gives pyruvic acid and l(+)-alanine in about 85 per cent yields. Valine, isoleucine, and methionine have also been resolved in this

manner. Resolution of synthetic dl-alanine by fractional crystallization of the strychnine salt of the benzoyl derivative has been reported (148). It seems to the authors that chromatographic absorption might be made use of in the resolution of amino acids just as it has been used in the resolution of other racemic materials.

Block (150) separates ammonia, arginine, histidine, and lysine from protein hydrolysates by the use of synthetic ion exchange resins (Amberlites IR-1, IR-100, and IR-14 prepared by Resinous Products and Chemical Co.).

6. Charcoal Adsorption. Tiselius (149) has reported some preliminary studies on the adsorption of amino acids and peptides by activated charcoal. The data are given in Table I. While charcoal adsorption may find some application in the isolation of amino acids, the results are not sufficiently clear-cut for quantitative use.

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SECTION II. THE PREPARATION OF AMINO ACIDS AND PROTEINS

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- 7. Naturally Occurring Amino Acids. (a) l(-)-Proline. The isolation of proline from protein hydrolysates by the use of rhodanilic acid as described by Bergmann (see Chapter III, p. 167) has been found satisfactory in the hands of Mayeda (131). However, if l(-)-hydroxyproline is to be isolated as the Reinecke salt according to the Bergmann procedure, it is first necessary to remove lysine which otherwise will be a contaminant of hydroxyproline (131).
- (b) l(-)-Methionine. Before attempting to isolate methionine, it is advisable to read a paper by Toennies and Kolb (132) in which they report the factors governing the precipitation of this amino acid with mercuric chloride. Methionine appears to form a mercuric chloride addition compound of the normal mercuric salt. Complete precipitation of methionine is favored by neutrality, by the absence of chloride ion (mercuric acetate may be used for this purpose), and by the presence of alcohol. The basic amino acids, which form precipitates with mercuric chloride, and the dl-carboxylic amino acids, which form precipitates with mercuric acetate, should be absent when methionine is to be precipitated as the complex mercury salt. The common other neutral amino acids do not interfere by precipitate formation.

The reactions between methionine and mercuric chloride appear to be:

$$2CH_3 \cdot S \cdot (CH_2)_2 \cdot CH(NH_3^+) \cdot COO^- + HgCl_2$$

$$\rightarrow [CH_3 \cdot S \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COO]_2 \cdot Hg + 2H^+ + 2Cl^-$$

$$(1)$$

and

$$\begin{array}{l} (\mathrm{CH_3 \cdot S}(\mathrm{CH_2})_2 \cdot \mathrm{CH}(\mathrm{NH_2}) \cdot \mathrm{COO})_2 \cdot \mathrm{Hg} + 4\mathrm{HgCl_2} \\ \longrightarrow [(\mathrm{CH_3 \cdot S} \cdot (\mathrm{CH_2})_2 \cdot \mathrm{CH}(\mathrm{NH_2}) \cdot \mathrm{COO})_2\mathrm{Hg}](\mathrm{HgCl_2})_4 \end{array}$$

With the continued liberation of H+ as in reaction (1), the equilibrium

 $\mathrm{CH_3 \cdot S \cdot (CH_2)_2 \cdot CH(NH_3^+) \cdot COO^- + H^+}$

 $\rightleftarrows \mathrm{CH_3 \cdot S \cdot (CH_2)_2 \cdot CH(NH_3^+) \cdot COOH}$

is increasingly shifted to the right. It is to be expected from equation (2) that mercuric acetate will form no precipitate with methionine in the absence of chlorides. Sodium chloride first increases precipitate formation and, on further addition, decreases it as would be expected from the following reactions:

$$\begin{split} (\mathrm{CH_3COO})_2\mathrm{Hg} + 2\mathrm{Cl}^- &\rightarrow \mathrm{HgCl_2} + 2\mathrm{CH_3COO}^- \\ \mathrm{HgCl_2} + 2\mathrm{Cl}^- &\rightarrow (\mathrm{HgCl_4})^- \end{split}$$

- (c) l(-)-Leucine is usually contaminated with methionine. Bergmann and Stein (133) prepare pure leucine by dissolving 100 gm. of leucine in 825 cc. of N HCl, heating, and then adding a hot solution of naphthalene-β-sulfonic acid dissolved in 175 cc. of water. After standing 24 hours at 0°, the crystals are filtered off and recrystallized successively from 900, 700 and 550 cc. of water. The salt is dried at room temperature and then suspended in 900 cc. of absolute ethanol. 180 cc. of pyridine are added. After standing several days at room temperature, the leucine is filtered off, washed with absolute ethanol, and resuspended in 900 cc. of absolute ethanol and 135 cc. pyridine. After standing a day, the leucine is filtered off, washed with absolute ethanol and ether, and dried. The leucine is next recrystallized by dissolving it in 1600 cc. of water, adding 800 cc. of ethanol, and permitting the mixture to stand at 0° for several days. The crystals are filtered off, washed with a little ice water, and dried. The yield was about 59 per cent of a commercial preparation that contained 88 per cent of leucine.
- (d) l(-)-Phenylalanine has been prepared by Baptist and Robson (134) by taking advantage of the fact that over the pH range of 4 to 7 this amino acid and leucine are very much less soluble in saturated sodium chloride solution than in water. Methionine is also less soluble in saturated sodium chloride solution than in water but more so than leucine and phenylalanine. After hydrolysis of the protein (zein) and adjustment of the hydrolysate to pH 6 with sodium hydroxide, tyrosine and leucine are crystallized out in the cold. The filtrate is concentrated until sodium chloride begins to separate. The mass of amino acids that separates in the cold is filtered off and washed with ice water to remove most of the sodium chloride. The amino acids are dissolved in water and further fractions of amino acids are removed by crystallization. This process is continued until small amounts of phenylalanine begin to crystallize out as shown by the Kapeller-Adler test. The amino acids in the filtrate, which contain most of the phenylalanine, are converted

into the copper salts. The copper salts, after repeated extraction with cold water, are decomposed with hydrogen sulfide and the phenylalanine is crystallized out as the picrolonate. The yield of phenylalanine picrolonate from 500 gm. zein is about 6 gm. Picrolonic acid may also be used to isolate phenylalanine from the butyl alcohol extract of a protein hydrolysate after first converting the amino acids into the copper salts and following the subsequent procedure given above. Phenylalanine picrolonate melts at 192° with decomposition. Methionine picrolonate melts at 178–180°.

8. Proteins. The isolation of proteins in a pure state is still, in most instances, beset with difficulties. So-called pure proteins are often characterized by modes of preparation rather than as chemical entities. By use of the electrophoretic method developed by Tiselius (see Chapter XII of the Addendum), it has been shown that some of the protein preparations that at one time were regarded as being reasonably pure, are actually mixtures of a number of closely related proteins. Thus serum globulin consists of α -, β -, and γ globulins. Casein has likewise been shown to consist of three fractions (135). The phosphorus content of the fastest moving fraction is greater, that of the slowest portion is smaller than the total phosphorus content of the casein. Repeated recrystallization of the protein preparation, when crystallization is possible, cannot always be taken as a criterion of purity since one must keep the possibility of mixed crystals in mind. If and when the electrophoresis technique is extended to the use of large quantities of protein solutions,1 it should be possible to obtain many more protein preparations that are reasonably pure than are now available. For the present, as many criteria as possible should be applied to protein preparations before accepting them as consisting of a single type of molecule. Chemical analysis alone is not a sufficient criterion of homomolecularity. Tests such as solubility, diffusion, sedimentation, osmotic pressure, mobility during electrophoresis, penetration through membranes, and change of dielectric constant, that are functions of molecular size and net charge, combined with crystalline homogeneity and chemical analysis, may be used for the present to characterize proteins. However, if one considers the possibility that proteins, when present in tissues, may be undergoing metabolic changes of an order that may not necessarily involve cleavage of amino acids, but rather splitting off of one or

¹ T. B. Coolidge (*J. Biol. Chem.*, 127, 551 (1939)) has designed a simple cataphoresis apparatus in which he separated some of the blood proteins.

more free groups, then even these methods may not prove to be sufficiently sensitive to distinguish between proteins that differ only slightly in their chemical makeup.

Contaminating salts, not in chemical combination, may be removed from protein solutions by dialyzing against distilled water and then electrodialysis in a three compartment cell, the compartments being separated by cellophane membranes. A simple apparatus in which mercury forms the electrodes is described by Joseph (136). A larger cell may be constructed of bakelite and carbon electrodes used. A slow stream of distilled water should circulate through the end compartments.

Isolation of proteins from tissues often presents a problem. The tissues may be frozen by immersion in liquid air or treatment with solid carbon dioxide before grinding and then extracted with a suitable solvent. Protein preparations may be kept unaltered for extended periods of time by evaporation of the solvent at very low temperatures and in high vacuum (lyophile method). Greaves and Adair (137) have described an apparatus for drying large quantities of proteins. Adair and coworkers (138) have shown that such drying does not lead to denaturation or change in some of the physical chemical properties that were examined.

Due to the present day difficulties of securing hemp seed which is used for the preparation of edestin, Vickery and coworkers (139) have studied the globulins from some of the common species of Curcubita. These proteins resemble edestin and will probably be useful as substitutes.

(a) Serum Proteins. The fractionation of blood serum, particularly the separation of the globulins from the albumins, may be effected by dialysis or electrodialysis at reactions close to the isoelectric points of the respective proteins, or by salting out the particular protein by suitable concentrations of such salts as sodium or ammonium sulfate and sodium or potassium phosphate. Of these, ammonium sulfate is most commonly employed. Cohn and coworkers (140) have carried out extensive studies on the fractionation of horse serum proteins with ammonium sulfate. Table I gives the proteins that are precipitated by various concentrations of ammonium sulfate.

Further purification of the proteins was effected by dialysis and repeated precipitation with ammonium sulfate (the reader should consult the original articles for the details). The γ -globulin proved to be the easiest to prepare in a fairly pure state. The electro-

phoretic mobility of a 1 per cent solution of this protein at 4° in phosphate buffer of ionic strength 0.2 and pH 7.7 is 1.9×10^{-5} . The sedimentation constant, $S_{20}^{1\%}w$, is 6.2×10^{-13} or 6.5×10^{-13} at infinite dilution. On the basis of a diffusion constant of 4.1×10^{-5} and a partial specific volume of 0.730, the molecular weight was calculated to be 142,000. The β -globulin fraction contained about 5 per cent of a protein that had a migration velocity about the same as that of γ -globulin. The electrophoretic mobility of the β -globulin is 2.9×10^{-5} . The mobility of the α -globulin fraction is about 3.9×10^{-5} .

TABLE I
Fractionation of Horse Serum Proteins

Conc. (NH ₄) ₂ SO ₄ moles/liter	Per cent of satura- tion	Type of protein precipitated	Per cent of total protein pre- cipitated		of protein pitated Water insoluble
1.39	0.34	Largely γ-globulin.	20	71	29
1.64	0.40	γ -, β -, α -globulin.	15	67	33
2.05	0.50	β-, α-globulin, muco- globulin.	14	94	6
2.57	0.62	Largely crystalline albumins.	32	98	2
2.80	0.68	Crystalline albumins, haemocuprein, cho- line esterase, glyco- protein, phosphatase.		99	1

(Cohn, E. J., McMeekin, T. L., Oncley, J. L., Newell, J. M., and Hughes, W. L., J. Amer. Chem. Soc., 62, 3386 (1940)).

The α -globulin preparation also contained small amounts of the other serum proteins. These experiments are indicative of the difficulties that are inherent when attempting to separate proteins whose properties are quite similar. They likewise indicate that no single criterion is usually sufficient for determining the homogeneity of protein preparations.

Cohn and coworkers (141) have also fractionated bovine serum by equilibration through cellophane membranes with ethanol-water mixtures of controlled pH, ionic strength, and at -5° to prevent denaturation. Four fractions were obtained. At 0° and 15 per cent ethanol, the precipitate consists largely of fibrinogen and fibrin together with small amounts of γ -globulin. On increasing the ethanol concentration of the filtrate to 20-25 per cent and at -5° ,

a precipitate consisting largely of γ -globulin was obtained. α - and β -globulins separated when the ethanol concentration was raised to 30–40 per cent. The filtrate from this fraction, in the presence of acetate buffer of ionic strength 0.05 and at pH about 5.5, contained chiefly albumin. The electrophoretic mobility of the bovine γ -globulin at 4° in phosphate buffer of ionic strength 0.2 and pH 7.7, is 1.6×10^{-5} or slightly less than that of horse γ -globulin. The sedimentation constant, $S_{20}^{1\%}, w, = 6.4\times10^{-13}$, is not very different than that of the horse γ -globulin. The values for bovine albumin are: electrophoretic mobility, 5.2×10^{-5} ; $S_{20}^{1\%}, w=4.0\times10^{-13}$. The

Table II

Constants Defining the Properties of Horse Serum Albumins

	Nitrogen	Optical rotation	Sedimen- tation constant	Electro- phoretic mobility pH 7.7
	per cent	$(lpha)^{20}D$	$S_{20}^{\circ}, w imes 10^{18}$	$\tau/2 = 0.2$ $\mu \times 10^5$
Crystalline unfractionated				
carbohydrate-free albumin	16.1	-57	4.1	5.3
Crystalline albumin sepa-				
rated as sulfate	16.8		4.1	
Crystalline haemocuprein	14.4			
Crystalline carbohydrate-				
containing albumin	15.1	-47	4.1	4.5

(McMeekin, T. L., J. Amer. Chem. Soc., 62, 3393 (1940)).

molecular weight of the albumin is half that of the γ -globulin and the net charge is half again as great.

The albumin fraction of horse serum can also be fractionated by repeated precipitations with ammonium sulfate. The salt concentration used by McMeekin (142) was 2.05-2.57 M. A homogeneous crystalline serum albumin containing 5.5 per cent carbohydrate and a carbohydrate-free albumin were obtained. The constants defining the properties of these albumins are given in Table II. A review of plasma proteins is given by Cohn (158). It should be emphasized here that much more reliability can be placed on data obtained on proteins that have a homogeneous crystalline structure than on those that do not.

(b) Hemoglobin. Due to its apparent homomolecularity and ease

of crystallization, especially when prepared from horse blood, this protein has been extensively investigated. Purification of horse hemoglobin has been attempted (143) by absorption on γ-Al(OH)₃ cream. The percentage saturation of these preparations with oxygen was higher than of whole blood. There is no minimum effect on the percentage saturation at 1 mm. of oxygen pressure within the pH range of 5.5-8.7. Two zones were obtained in a Tswett column, suggesting the possibility of two forms of hemoglobin. However, on elution with phosphate buffer and in the presence of air, both layers were identical spectroscopically. The suggestion is made that hemoglobin is either in a different form when present in the erythrocyte than that obtained by the use of Al(OH)3, or is associated with some other substance. The pH of the hemoglobin solution changes but little with oxygenation. It appears probable that two acid groups on each heme are affected by oxygenation. On this basis the pK values in the reduced and oxygenated conditions are respectively 6.5 and 5.8, and the value for K", the oxygen association constant for unionized hemoglobin, has a value of 0.024 (144). The value of ΔH for the reaction between oxygen and hemoglobin is -15,500 calories per mole of oxygen, and ΔF° for the reaction between the first oxygen molecule and hemoglobin is -3000calories per mole.

The preparation of *myoglobin* is given by Theorell (145) and *carboxyhemoglobin* by Green, Cohn, and Blanchard (146).

(c) Insulin. In the preparation of insulin, advantage is taken of the fact that this hormone is soluble in a sufficiently high concentration of ethanol so that when fresh minced pancreas is treated with acidified ethanol, the proteolytic enzymes and some of the tissue proteins are not solubilized. By adjusting the acidity of the crude extract to pH 6.6 or slightly more alkaline, it is possible to precipitate proteins whose isolectric points are close to pH 7.0 and still keep the insulin in solution. Similarly, adjustment of the filtrate to pH 3.5 will precipitate proteins whose isolectric points are in this vicinity. On adjusting the pH of this filtrate to pH 5.0, crude insulin will separate. The isolectric point of insulin is 5.2. At pH 3.8 and at pH 6.6, some insulin will separate. Maximum yields are, however, obtained only at, or close to the isolectric point. Further purification is effected by repeatedly salting the protein out with sodium chloride at pH 2.5, reprecipitation at pH 5.0, removal of foreign proteins by addition of alcohol to the insulin solution at pH 2.5, and addition of sufficient alcohol and ether to precipitate the hormone. The product is dried in vacuo. The product still contains impurities. It may be purified (147) by dissolving it in 0.05M KH₂PO₄, adding a solution of zinc chloride, and then isopropyl alcohol to a concentration of 10 per cent. On adjusting the pH to 6.8 and heating the mixture to 50°, a precipitate is formed. The pH of the filtrate is brought to 6.5. On standing at low temperature, crystals of insulin separate.

Sahyun (148) has prepared crystalline insulin which contained 0.149 per cent of zinc, 15.5 per cent of nitrogen, 2.96 per cent sulfur, 3.0 per cent moisture, and 0.55 per cent ash. It also contained traces of cobalt, nickel, and cadmium. The crystals are rhombohedral. The presence of small amounts of heavy metal such as zinc, markedly slows the rate of deterioration of the hormone. Cohn and coworkers (159) prepared radioactive zinc insulin. In systems of low ionic strength acid to pH 5.5, the crystals contained about 0.34 per cent radioactive zinc. The solubility of zinc insulin at 5° is about 0.01 gm. per liter.

(d) Lack of space prevents further brief descriptions of the isolation of other proteins. References to a few of the recently described methods are: thrombin (149), phytothrombin (150), skeletal muscle albumin (151), myogens from rabbit muscle (152), fibrinogen (153), casein fractions (154), gluten fractions (155), and flavoprotein (156).

References to the methods employed for the isolation of virus proteins are given by Stanley (157).

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CHAPTER IV

METHODS OF ANALYSIS AND REACTIONS OF THE AMINO ACIDS AND PROTEINS

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- 1. Introduction. In recent years a number of theories of protein structure has been proposed. Many of these theories are dependent upon the quantitative amounts of amino acids in the particular protein discussed, so that it has become necessary to find more accurate methods of analysis in order to substantiate or disprove the proposed theories (76). It has also been necessary to analyze for certain amino acids in connection with some pathological conditions. Block and Bolling (77) have published detailed analytical methods for the majority of the amino acids.
- 2. Reactions of Amino Acids and Proteins. A study has been made of the use of the biuret reaction in the determination of serum proteins in an attempt to establish the optimum conditions necessary for the production of a stable color which will have a quantitative relationship to protein concentration (78). A comparison of this method with the Kjeldahl procedure indicates that it gives satisfactory results. Methionine will give a stable deep orangebrown color when treated with cupric chloride in concentrated hydrochloric acid (79). Methionine is the only known naturally occurring amino acid that gives this reaction. Homomethionine, hexomethionine, ethionine and homodjenkolic acid also give this reaction. It has also been reported that the addition of an excess of metaphosphoric acid to a protein will produce a precipitate in which the phosphorus content is equivalent to the number of positively charged groups of the protein (80).
- 3. General Quantitative Methods. The general quantitative methods for analysis of amino acids have been developed along three lines: microestimation methods, solubility methods, and physical methods. The formol titration has been adapted to a micro method (81). The apparatus and procedure for determination of small amounts of amino acids by the ninhydrin reaction has been

described (82). A microestimation method for leucine and valine based on the determination of the acetone obtained by the oxidation of the deaminized amino acid has been developed (83, 84, 85). Isoleucine is estimated by measuring the ethylmethylketone obtained from the oxidation of the corresponding hydroxy acid. A photometric method for the determination of tryptophane, tyrosine, diiodotyrosine and thyroxine has been developed from the Lugg modification of the Folin-Ciocalteau method (86). The iodoamino acids are converted into reactive phenols by alkaline stannite hydrolysis of the protein and they can then be indirectly determined by this method. Another photometric method for the determination of tyrosine, tryptophane, and cystine based on the colorimetric method of Folin-Marenzi has been reported (87).

The solubility method of Bergmann and Stein (88) makes use of the fact that the product of the ions of a saturated solution of a difficultly soluble salt is a constant. Thus, if the solubility product of a salt is known, and the concentration of one of the ions is known, the other may be calculated. The advantage of this method is that complete isolation is not necessary. The reagent can be added until some precipitate is formed, and then from the quantity added, from the weight of the precipitate, and from the solubility product, the total amino acid content can be calculated. This method has also been adapted to a semi-micro technique (89). The naphthalene- β -sulfonic acids (90) and several other aromatic sulfonic acids (91) that can be used in the solubility method have been investigated.

The isotope dilution method for the determination of amino acids (92) makes use of the rather neat principle that if a known amount of an isotopic amino acid is added to a mixture, and then some of the amino acid is isolated, the percentage of isotope in the isolated amino acid will bear a quantitative relationship to the total amino acid present in the mixture. The quantity originally present, y, can be calculated from the equation

$$y = \left(\frac{C_0}{C} - 1\right) x$$

where x is the amount of amino acid added, C_o = the isotope content above normal of the amino acid added, and C = the isotope content of the isolated amino acid. As more laboratories become equipped to work with isotopes, this method will probably find wider application. An electrical method for the estimation of the

basic amino acids has been developed on the basis that the basic amino acids appear in the cathode compartment when a current is passed through a protein hydrolysate (93). The advantage of this procedure is that humin, hydrochloric acid, and amide nitrogen do not interfere. Critical reviews and detailed analytic procedure are given by Tristram (94) and by Laine (95). The identification of amino acids by means of 3,5-dinitrobenzoylchloride has been extensively studied (96).

- 4. Some Special Methods for the Estimation of Individual Amino Acids. (a) Tyrosine. Tyrosine can be determined in the filtrate from a Foreman precipitate of a protein hydrolysate by oxidizing it with bromine, potassium bromide, and potassium permanganate (97). The oxidized product is distilled into dinitrophenylhydrazine. The precipitate thus formed is dissolved in pyridine. Water and sodium hydroxide are added, and the blue color thus produced is measured. A simplified modification of the Lugg procedure for tyrosine has been presented (98) and Lugg has reported the optimum conditions of hydrolysis for this determination (99).
- (b) Cysteine, Cystine, and Methionine. A colorimetric method for cysteine based on the color developed with para-aminodimethylaniline in acid solution in the presence of iron has been developed (100) and has been used as a micromethod by reading the color at 5800 A° with an error of ±3 per cent (101). A photometric determination of cystine and cysteine with phosphotungstic acid according to a modification of the Folin-Lugg method has been described, and the determination after hydrolysis with hydriodic acid is discussed (102). It has been shown that cuprous oxide can be used for the reduction and precipitation of cystine replacing zinc in the reduction (103). Thus, any possible zinc phosphate precipitate is avoided. A polarographic microdetermination of cystine has been developed. It is based on the observation that a characteristic polarographic reaction is given by cobaltous chloride in ammonia and ammonium chloride in the presence of cystine or compounds containing the sulfhydryl group (104). The reaction is not linear with respect to the concentration of cystine, but by using a calibration curve, very satisfactory results can be obtained. Kuhn et al. (105) have adapted the iodometric titration of sulfhydryl groups to a micromethod for the determination of cystine, cysteine, and methionine. They have found that iodine will dehydrogenate sulfhydryl compounds rapidly to disulfides in exact stoichiometric

proportions in 90 per cent acetic acid solution. Very small amounts of material can be used and oxygen need not be excluded. Detailed procedures for cysteine, cystine, and methionine are given. It has been suggested that use could be made of the hydrogen peroxide oxidation of methionine for an analytical method (106).

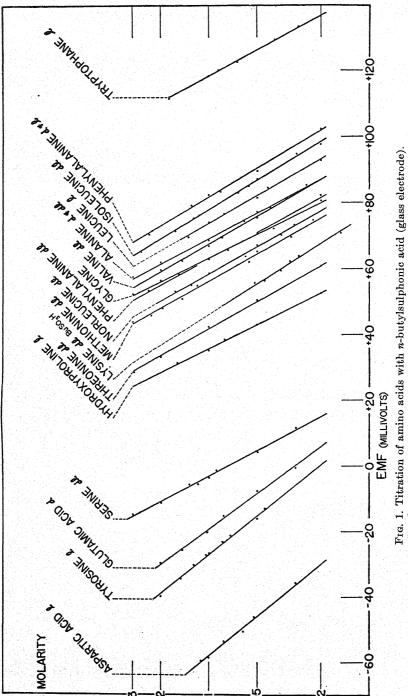
- (c) Tryptophane. The p-dimethylaminobenzaldehyde method for tryptophane has been adapted to a rapid procedure for estimating tryptophane in casein by employing a weak hydrogen peroxide solution (107). This method has also been critically compared with the glyoxylic acid method (108) and the reliability of the glyoxylic acid method confirmed (109). Lugg (99) has also investigated the sources of error in the estimation of tryptophane. Another colorimetric determination of tryptophane proposes the use of the color produced by heating the amino acid solution with a 2 per cent acid alcoholic solution of vanillin (110).
- (d) *Histidine*. Histidine can be estimated by precipitating it in 50 per cent methanol with solid nitranilic acid, after it has been recovered from a silver precipitate and the silver removed (111).
- (e) Arginine. By using excess α -naphthol and adjusting the sodium hypobromite concentration to give the most stable color, the Sakaguchi reaction gives quite satisfactory results for arginine (112). The optimum conditions for the use of flavianic acid in determining arginine has also been reported (113).
- (f) Glutamic Acid. The determination of as little as 0.1 mg. of glutamic acid can be carried out by converting it into succinic acid with chloramine T and determining the succinic acid manometrically by means of a succinoxidase preparation (114). Glutamine interferes but glutathione will not if precaution is taken to remove, by extraction with ether, any β -cyanopropionic acid formed. Glutamic acid can also be converted to succinic acid by permanganate oxidation of the deaminized glutamic acid, and the succinic acid so formed can be measured by the amount of silver necessary to form the salt (115).
- (g) Proline and Hydroxyproline. Proline has been determined by the solubility method using ammonium rhodanilate as the precipitating agent (116). Hydroxyproline can be estimated by oxidizing it with sodium peroxide in the presence of copper or cobalt and condensing the chromagen thus formed with isatin to form a stable red complex (117). Proline can likewise be oxidized using lead dioxide, and the oxidized product, when condensed with the oxidation product of p-dimethylaminobenzaldehyde, will give a red compound (118). Hydroxyproline interferes.

- (h) Serine and Threonine. Serine and threonine can be determined by isolating and measuring the formaldehyde and acetaldehyde formed respectively when these amino acids are treated with periodic acid (119, 120). The formaldehyde is determined as its dimedone derivative, and the acetaldehyde as its sodium bisulfite addition product. The recovery is good. Carbohydrates and hydroxylysine interfere in the serine determination. Threonine has also been oxidized with lead tetracetate to acetaldehyde which was determined colorimetrically with p-hydroxydiphenyl (121).
- (i) Other Amino Acids. Block's isolation procedure has been modified so that lysine can be quantitatively separated from arginine and histidine and precipitated as the picrate (122). Lysine has also been determined by precipitation with styphnic acid in 80 per cent alcohol with mineral acids absent from the filtrate of the flavianic acid precipitation of arginine (123). Leucine has been determined with a 5 per cent error by oxidizing it with ninhydrin to isovaleraldehyde which is measured as its dimedone derivative (124). Alanine can be determined by precipitation with dioxypyridic acid (dioxaltodipyridinochromiato acid) (125). Van Slyke, Hiller and Mac Fayden (126) determine hydroxylysine in protein hydrolysates by precipitating it together with the other diamino acids as the phosphotungstate and determining the ammonia evolved from the -CH(OH) · CH(NH₂)-group of the hydroxylysine when the diamino acids are treated with alkaline periodate. The reaction between sodium periodate and hydroxyamino acid is

$R \cdot CH(OH) \cdot CH(NH_2) \cdot R' + NaIO_4 \rightarrow R \cdot CHO + R' \cdot CHO + NH_3 + NaIO_3$

5. Gasometric Analysis. Van Slyke, Dillon, Mac Fayden, and Hamilton (127) determine free amino acids on the basis of the CO₂ evolved from the carboxyl groups when the amino acids are treated with ninhydrin. The gas is measured in the Van Slyke-Neill manometric apparatus. At a properly chosen pH, each known amino acid yields 1 mole of CO₂ except cystine and aspartic acid which yield 2 moles. Only one carboxyl group of glutamic acid reacts. In protein digests carboxyl determinations indicate the amounts of free amino acids formed. Peptides as a class yield no CO₂ or only traces. Glutathione, in which the -CH(NH₂)·COOH group is free, reacts. Crystalline trypsin digests casein to peptides without liberation of free amino groups. The CO₂ may also be determined by absorbing it with barium hydroxide and titrating the excess hydroxide (128).

Kendrick and Hanke (129) have shown that by adding potassium



(Rask, O. S., and Eckles, N. E., Amer. J. Hyg., 33 (sec. a), 86 (1941)).

iodide to the sodium nitrite in the Van Slyke method for estimating amino nitrogen, theoretical values for glycine and cystine are obtained both in the presence and absence of metallic mercury.

6. Titration of Amino Acids. All of the accepted amino acids except cystine and thyroxine are dissolved by *n*-butylsulphonic acid in 95 per cent acetone (130). The following are the moles of *n*-butylsulphonic acid required per mole of amino acid: aspartic acid, 1.63; tyrosine, 1.23; glutamic acid, 1.16; lysine, 2.11; arginine, 2.00; histidine, 2.00; tryptophane, 0.923; all others, 1.0. The titration of the amino acids with *n*-butylsulphonic acid may conveniently be carried out with the glass electrode. The titration curves are essentially straight lines (Fig. 1) and follow the equation

$$\log y = -mx + b$$

where y = molar concentration, x = glass electrode potential, b = a constant, characteristic of, and different for, each amino acid, and m = a constant (straight line) varying somewhat with each amino acid.

Amino acids may also be titrated with perchloric acid in acetic acid (131). The values are essentially in agreement (small deviations were found) with those obtained by potentiometric (glass electrode) formol titration (132).

7. Amino Acid Content of Proteins. The amino acid contents of a number of keratins including hair, wool, horn, gorgonin, spongin, turtle scutes, cow hair, and chimpanzee hair have been reported (133, 134, 135). Analyses for 17 amino acids in 4 marine algae using formic acid extracts have been made (136). The amino acid contents equivalent to 77 per cent of the weight of rabbit myosin have been characterized (137). The following values have been obtained for some of the more common proteins: gelatin: 17.5 per cent proline (116), 14.65 per cent hydroxyproline (117), 3.31 per cent serine, +hydroxylysine (119); silk fibroin: 13.57 per cent serine, 1.36 per cent threonine; silk sericin: 33.9 per cent serine, 8.9 per cent threonine (138); casein: 5.16 per cent serine, 3.50 per cent threonine; lactalbumin: 4.26 per cent serine (119); insulin: 3.57 per cent serine, 2.66 per cent threonine, 1.83 per cent other hydroxyamino acids (139). Vickery (140) has reported the amino acid content of zein. The arginine and histidine values are 1.99 and 0.88 per cent respectively. Zein contains no lysine. Analyses of pepsin (141) and chymotrypsinogen (142) have been reported. The cystine and methionine content of globin varies with the species from which the globin is obtained (143).

Cysteine, glutathione, and the substituent sulfhydryl group of denatured proteins may, under proper conditions, be estimated by treatment with o-iodosobenzoic acid (144). The reaction, in the case of cysteine, is

 $2 - OOC \cdot CH(NH_3^+) \cdot CH_2SH + C_6H_4(COO^-)IO \rightarrow$

 $-\mathrm{OOC}\cdot\mathrm{CH}(\mathrm{NH_3^+})\cdot\mathrm{CH_2}\;\mathrm{S}\cdot\mathrm{S}\;\mathrm{CH_2}\cdot\mathrm{CH}(\mathrm{NH_3^+})\cdot\mathrm{COO}^-+\mathrm{I}\;\mathrm{C_6H_4}\;\mathrm{COO}^-+\mathrm{H_2O}$

The estimation is carried out by adding 10 cc. of a 0.01812 N o-iodosobenzoate solution to 5 cc. of a molar phosphate buffer solution of pH 7 followed by 10 cc. of the cysteine hydrochloride solution (0.011 N). After 30 seconds an acidified solution of potassium iodide (0.5–1.0 gm. KI in 1.5 cc. H₂O, treated with 5 cc. of N HCl immediately before use), is added. The liberated iodine is titrated at once with standard sodium thiosulfate.

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CHAPTER V

THE RELATION OF THE AMINO ACIDS TO PRODUCTS OF BIOCHEMICAL IMPORTANCE

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It is not the purpose of this chapter to give the reader a detailed account of amino acid and protein metabolism in the animal body. However, so many new facts have been learned from metabolic experiments about the reactivity of certain groups in amino acids and proteins that a brief account appears necessary.

1. Isotopes as Metabolic Indicators. The ideal manner for studying the metabolism of amino acids and proteins would be to follow the fate of every atom in a particular amino acid or protein from the time that the substance is fed until all of the atoms in the molecule are accounted for in the metabolic end products. With the production of isotopes, such a technique at least in part, has become available. Two types of isotopes for "tagging" amino acid molecules can be used. These are the radioactive (33) and the stable types. Of the former, the long lived radioactive carbon, C¹⁴, is not yet generally available in sufficient quantities for synthetic purposes (34) and the short lived isotope, C11, with a half life of 21 minutes is usually not adapted for metabolic experiments of the type that necessitate synthesis of amino acids in vitro before feeding experiments can be undertaken (35). Radioactive sulfur, S35, with a half life of about 80 days, is so adapted. Tarver and Schmidt (36) incorporated this isotope into the methionine molecule. The methionine was fed to young rats. After a period of time, cystine containing S35 was isolated from the hair of these rats. The tentative conclusion is that methionine was converted to cystine. It is possible that methionine, on demethylation, is converted into homocysteine. The latter compound, on loss of a -CH2 group would yield cysteine. On the other hand it is likewise possible that the -SH group of homocysteine can shift to some other molecule such as serine or pyruvic acid and thus yield cysteine. It will be necessary next to show whether or not the carbon atoms in cystine are the same as those that were contained in methionine. If not, then it will be necessary to determine to which compound either the $-SCH_3$ group of methionine or the -SH group of homocysteine is transferred. Unpublished experiments by Tarver and Schmidt show that taurine also contains S^{35} when methionine containing this isotope is fed to dogs (60).

When the long lived radioactive carbon and radioactive hydrogen or tritium become generally available, it will be possible to use them to tag amino acids. Radioactive iodine can be used as a tag for the iodine-containing amino acids (62).

The stable isotopes that have been incorporated into amino acid molecules are heavy hydrogen or deuterium and isotopic nitrogen, N¹⁵ (37). It is possible that in the future heavy oxygen² and sulfur may also be employed. One advantage in using the stable isotopes is that one is not confronted with the problem of diminishing activity. Heavy hydrogen, when present in organic molecules, can be determined as heavy water after combusion. Isotopic nitrogen is estimated with the aid of the mass spectrograph. As little as 0.004 per cent N¹⁵ can be determined. In other words, it is possible to synthesize an organic compound with about 5 per cent N¹⁵ in excess over that occurring naturally, mix it with 1000 times its natural, non-isotopic analogue, and still determine the N¹⁵.

2. Synthesis of Isotopic Proline. For metabolic purposes amino acids may be regarded as consisting of two parts, the carbon chain and the nitrogen-containing group. The carbon chain may be tagged by incorporating deuterium while the nitrogenous group is marked with N¹⁵. The tagging of an amino acid with these isotopes may be illustrated by the synthesis of proline³:

¹ Since this was written it has been shown that the carbon chain of serine is converted to cystine (61).

² Heavy oxygen exchange reactions of amino acids and proteins have been reported by Mears, W. H., and Sobotka, H., J. Amer. Chem. Soc., 61, 880 (1939).

³ Note: N¹⁵ is marked by a star and heavy hydrogen by a small circle.

$$\begin{array}{c} \mathring{H}_{2} \\ \mathring{C} \\ \mathring{C}$$

Since only the naturally occurring isomeric form of many of the amino acids is used by the animal body, resolution of the racemic compound is necessary. The *l*-form of the amino acid may usually be obtained by treating the racemic amino acid with *d*-amino oxydase, whereby the *l*-amino acid, the isotopic keto acid, and isotopic ammonia are obtained. These may be easily separated. Certain amino acids, such as glutamic acid and lysine, are resistant to the action of *d*-amino oxydase and the usual chemical methods for resolving the isomers must be employed.

3. Metabolism Experiments. For the purpose of feeding experiments, small amounts of the isotopic amino acid are included in the diet of small animals such as the rat or the pigeon. The excreta are collected and analyses for the isotope-containing compounds are carried out. The tissues of the animal may similarly be analyzed. Table I is illustrative of the data obtained.

Table I

Fate of Amino Nitrogen in Normal Adult Rats¹

		Per cent of administered N ¹⁵ recovered		
		After feeding $l(-)$ -leucine	After feeding glycine	
Excreta	Feces	2.2	2.6	
	Urine	27.4	40.8	
Animal body	Nonprotein nitrogen	8.2	11.1	
	Protein nitrogen	56.5	44.5	

¹ Isotopic amino acids, corresponding to 25 mg. nitrogen per day for five days, were added to normal stock diet.

(Schoenheimer, R., Protein Symposium, Stanford University, June, 1941.)

Table II Biological Nitrogen Transfer in Proteins

 N^{15} content of amino acids isolated from proteins of rats given l-(-)-leucine and glycine. (Calculated for 100 atoms per cent N^{15} in compound administered.)

	Li	Liver		Intestinal wall		Muscle and skin	
Amino acids	Leucine	Glycine	Leucine	Glycine	Leucine	Glycine	
Leucine	7.95		7.95		1.90		
Glycine	0.74	8.88	0.63	4.23		1.05	
Tyrosine	0.50	0.46	0.94		0.20	0.13	
Glutamic acid	1.85	0.89	2.97		0.89	0.27	
Aspartic acid	1.16	0.73	2.30		0.70	0.20	
Arginine	0.89	0.78	0.43		0.25		
Lysine	0.06		0.07			0.09	
Amide nitrogen	0.78	1.45	1.24			0.51	

(Schoenheimer, R., Protein Symposium, Stanford University, June, 1941.1)

It is evident from the data that the major portion of the N¹⁵ was present in tissue proteins. Similar results were obtained with most of the other amino acids and also with ammonia when these were fed. In the case of leucine, the experiments indicate that the isotopic amino acid of the diet had replaced a certain portion of the leucine that was present in tissue proteins. However, smaller amounts of the N¹⁵ were also contained in other amino acids that were isolated and particularly the dicarboxylic amino acids, indicating that the nitrogen of leucine was transferred to other carbon chains. (See Table II.)

The transfer of N^{15} from leucine to other amino acids is not peculiar to this amino acid. It is quite a general phenomenon and applies to other amino acids as well. Thus when isotopic tyrosine was fed, N^{15} could be isolated from the α -amino group of histidine. It was not, however, present in the imidazole ring. When isotopic phenylalanine was fed, isotopic tyrosine was isolated. Lysine apparently is incapable of accepting isotopic nitrogen from other amino acids when these are fed. Once nitrogen is removed from lysine, the deaminated carbon chain is not reaminated. The continuous deamination and amination of amino acids can be convincingly demonstrated by feeding doubly marked l(-)-leucine,

$$\mathring{H}_3\mathrm{C}$$

$$C\mathring{H}\cdot C\mathring{H}_2\cdot \mathrm{CH}(\mathrm{N^*H_2})\cdot \mathrm{COOH}$$

$$\mathring{H}_3\mathrm{C}$$

¹ See also Schoenheimer, R., The Dynamic State of Body Constituents, Cambridge, 1942.

If the amino group of leucine were not removed and replaced by amino groups from other amino acids, it would be expected that a constant ratio between the stably bound deuterium and the N^{15} of leucine isolated from tissue proteins should exist. This was not the case. The D/N^{15} ratio increased indicating that the N^{15} had been replaced by non-isotopic nitrogen.

The amino group of glutamic acid is exceptionally active metabolically. Thus when α,β -deutero- α -N¹⁵-glutamic acid was fed, no deuterium was present in the glutamic acid isolated from tissues, and only small amounts of N¹⁵, not more than could be expected if it had become detached and then reattached to the glutamic acid molecule. Glutamic acid present in proteins is likewise rapidly metabolized. The high metabolic activity of the glutamic acid in proteins may be the result of the participation of its corresponding α -keto acid in metabolic cycles.

When deutero ornithine was fed to mice, the deuterium appeared in arginine, proline, and glutamic acid, indicating that these amino acids were synthesized from ornithine. When N¹⁵ was incorporated into the guanidino group of arginine and the labelled amino acid fed to rats, isotopic nitrogen was present in urea. According to the Krebs cycle and in line with the above, ornithine can be reconverted in part to arginine and in part to proline and glutamic acid. Presumably ornithine would in part be metabolized to carbon dioxide and ammonia.

When isotopic glycine was fed to rabbits immunized against pneumococcus antigen, N¹⁵ was found to be present in the amino acids isolated from the various fractions of blood serum including the specific antibody. These results indicate that the antibody as well as the other serum proteins are undergoing constant metabolic change.

The above experimental data can be interpreted as follows: (a) the lability of the amino group is indicated. If amino acid A tagged with N¹⁵ is fed and the isotope is isolated from amino acid B, it is evident that transamination has taken place (or deamination and reamination). The name transaminases has been suggested for the enzymes concerned in this reaction. (b) If the N¹⁵ is contained in a peptide linkage, it is necessary to assume that a great many peptide linkages are opened and closed. Whether or not this signifies total or only partial destruction and resynthesis of protein molecules is not yet certain. At any rate the reactions involved proceed quite rapidly. Perhaps the greatest use of the isotopic technique will be found in determining rates of metabolism.

4. Ammonia. Ammonia is formed by deamination of amino acids and other compounds that contain the amino group. Most of the ammonia is converted into urea. However, as indicated earlier, some of the ammonia may be used for purposes of reamination of keto acids. Du Vigneaud and Irish (38) have presented evidence in favor of the idea that acetylation is an essential part of this reaction. In its simple form the reaction proceeds as follows:

$$\begin{array}{cccc} R & R \\ | & | & | \\ CH_2 & CH_2 + NH_3 \\ | & | & | \\ CH(NH_2) & C=0 \\ | & | & | \\ COOH & COOH \\ Amino Acid & Keto acid \end{array}$$

5. Inversion of Amino Acids. A scheme to account for the transfer of isotopic nitrogen is given by du Vigneaud and coworkers (39). Like the other reactions it takes into account the rôle played by ammonia and the keto acid.

In support of the above reactions du Vigneaud and coworkers showed, by the use of N¹⁵, that most of the original nitrogen of d-phenylaminobutyric acid that was fed was replaced by new nitrogen. An alternative hypothesis based on the formation of the Schiff base is also presented:

- 6. Transamination. Cohen (40) has presented evidence for the participation of keto acids in transamination. The transaminase contained in pigeon breast and pig heart muscle catalyze reactions (1), (2), (4), and (5) but not reaction (3).
- (1) l(+)-Glutamic acid+oxaloacetic acid $\frac{a}{b}\alpha$ -ketoglutaric acid

+l(-)-aspartic acid.

(2) l(+)-Glutamic acid+pyruvic acid $\stackrel{a}{\rightleftharpoons} \alpha$ -ketoglutaric acid

+l(+)-alanine

- (3) l(-)-Aspartic acid+pyruvic acid $\underset{b}{\rightleftharpoons}$ oxaloacetic acid+l(+)-alanine
- (4) l(-)-Cysteic acid $+\alpha$ -ketoglutaric acid $\overset{a}{\rightleftharpoons}l(+)$ -glutamic acid

 $+\beta$ -sulfopyruvic acid

(5) l(-)-Cysteic acid+oxaloacetic acid $\stackrel{a}{\rightleftharpoons} l(-)$ -aspartic acid

 $+\beta$ -sulfopyruvic acid

Experimental evidence for reaction (4b) and (5b) is not yet available.

Reaction (1a) proceeds at the fastest rate in all the tissues studied (heart muscle, skeletal muscle, brain, liver, kidney, testis, lung, and spleen). Reaction (1b) proceeds at a rate one-half to one-third that of reaction (1a) in the different tissues. Reactions (2) and (3) are very slow when compared with reaction (1).

The possibility exists that phosphorylation may play a rôle in amination and protein synthesis as the following reactions indicate (41):

7. Creatine Synthesis. Not all of the nitrogen of the guanidino group of arginine is ultimately excreted as urea. A part is converted to creatine in accordance with the following reactions:

The above reaction finds support from the following experiments: (a) Borsook and Dubnoff (42) have shown that liver slices of cat, rabbit, and rat convert glycocyamine (guanidinoacetic acid) to creatine. The methylation in rat liver is accelerated by methionine. (b) Schoenheimer and coworkers (43) have demonstrated by feeding isotopic arginine, $H_2N^*\cdot C:(N^*H)\cdot NH\cdot (CH_2)_3\cdot CH(NH_2)\cdot COOH$, and isotopic glycine, $N^*H_2\cdot CH_2\cdot COOH$, that only the isotopic nitrogen of these amino acids is used for the synthesis of creatine. The formation of guanidinoacetic acid is a slow process while the subsequent methylation reaction proceeds rapidly. The origin of the methyl group is discussed in the next section. While the precursors of creatine are established with great certainty, the chemical reaction whereby the guanidino group is split from arginine is not yet clear.

8. Transmethylation. Du Vigneaud and associates (44) fed methionine, which contained a deutero-methyl group, to rats and were able to isolate deuterocholine and deuterocreatine from the tissues. The deuterium was contained in the methyl groups of both compounds. The experiments show that methionine contributed the methyl group for the methylations. Similarly, deuterocreatine was isolated from the tissues of rats that were fed a diet in which deuterocholine and homocystine were included. Evidently the methyl groups of choline are first used for the synthesis of methionine, probably by methylating homocystine. The animal appears not to be able to synthesize the methyl group and it is therefore quite likely that either methionine or choline or both serve as the source of the methyl groups of adrenaline, ergothioneine, anserine, and other methyl-containing compounds. Creatine does not act as a methylating agent. Choline is essential for the prevention of fatty infiltration of the liver and hemorrhagic kidneys (45). A dietary supply of labile methyl groups is therefore essential. In this respect methionine has an important function. The chemical reaction concerned in transmethylation or the factors that labilize the methyl group have not yet been made evident by experiment. It is possible to imagine, since nitrogen is more negative than sulfur, that as the amino group comes in close proximity to the $-SCH_3$ group in methionine, the sulfur bond is labilized with the result that the methyl group shifts and methylation of the amino group occurs.

- 9. Uric Acid. Pigeons fed isotopic ammonium salts excrete isotopic uric acid. The N¹⁵ concentration in the nucleic acids obtained from the internal organs was very high. The two purines, adenine and guanine, contained more N¹⁵ than can be accounted for by the free amino groups and hence the N¹⁵ must be present in the ring (37). The experiments not only indicate that ammonia may be used for purine synthesis but also that the nitrogen of the purines is quite labile.
- 10. Resumé. It has become more and more evident that none of the components of living matter is in a static state. In fact all chemical compounds are labile, the degree of lability depending on the chemical nature and probably also the activity of the tissue in which the compounds occur. It becomes necessary to regard living tissues as being made up of many components that are in a state of dynamic equilibrium. In this respect, proteins belong to the most active. Schmidt, Allen, and Tarver (46) have pointed out that it is easily conceivable that proteins may undergo slight structural changes that do not involve degradation to amino acids and thus one protein may be transformed into another protein.

The student should realize that when reactions are written that involve proteins and amino acids, especially those reactions that represent metabolic processes, it is probably not possible at the present time and in most instances to write the complete chemical reactions since this would require the inclusion of all compounds that are formed or participate in the reaction however transitory they may be. Our knowledge of these reactions is largely based on the compounds that have been isolated and are relatively stable. The difficulties in isolating transitory compounds are evident. The remarkable thing about amino acids and proteins, metabolically speaking, is the ease with which groups may be shifted. This is especially so when consideration is given to the relative stability of the amino acids in vitro. Even though tremendous progress has been made in our understanding of the reactivity of amino acids and proteins in vivo, our knowledge of this subject will not be complete until the mechanisms, rates, and factors that are concerned in labilizing groups are known.

- 11. Glycogenic Amino Acids (page 223). The experiments of Butts and coworkers (47) has shown that dl-phenylalanine, dlserine, dl-valine, dl-tyrosine (slightly), l(+)-arginine, l(-)-tyrosine, glycine, d- and dl-alanine, d-glutamic acid, l- and dl- aspartic acid, dl-pyroglutamic acid, dl-norleucine, and dl-isoleucine form glycogen when fed to rats while dl-isovaline, dl-lysine, dl-leucine, and l-cystine do not. dl-Leucine gives rise to acetone while dl-lysine does not. Under certain conditions (feeding it as the sodium salt or with acetoacetic acid) dl-isoleucine may form acetone. When dlphenylalanine, but not dl-tyrosine, was fed at a level of 28.6 gm. per sq. m. of body surface, homogentisic acid was excreted. In this connection the experiments of Sealock and Silberstein (48) are of particular interest. When tyrosine was fed to guinea pigs deficient in ascorbic acid, homogentisic, p-hydroxyphenylpyruvic, and p-hydroxyphenyllactic acids were excreted. The administration of about 10 mg. daily of l-ascorbic acid prevented the excretion of these compounds.
- 12. Mercapturic Acids (page 236). p-Chlorophenol, when fed, does not lead to the excretion of a mercapturic acid. Hence it does not appear that the p-halogen phenols constitute an intermediary step in the synthesis of mercapturic acids when, for example, bromobenzene is fed (49). Benzene, chlorobenzene, o- and m-dichlorobenzene yield mercapturic acids while toluene, o-chlorotoluene, p-chloroacetanilide, p-chloroanisole, the corresponding o- and m-compounds, o- and m-chlorophenol, and phenol do not. In more recent times, it has been shown that the three monohalogenated benzenes, naphthalene, anthracene, benzyl chloride, p-bromobenzyl bromide, p-bromobenzylcysteine, and p-bromobenzylglutathione are partially converted in vivo to the corresponding mercapturic acids (50).
- 13. Pantothenic Acid. The synthesis of this compound has been carried out (51). Pantothenic acid consists of $(+)\alpha,\gamma$ -di-hydroxy- β,β' -dimethylbutyric acid joined with β -alanine by means of a peptide linkage:

$$\begin{array}{ccc} CH_3 & OH \\ & & | \\ HOCH_2 - C - CONH \cdot CH_2 \cdot CH_2 \cdot COOH \\ & | \\ CH_3 \end{array}$$

This compound, which is classed as a vitamin, when fed to chicks promotes growth and prevents (or cures) a specific dermatitis (52).

Hydroxypantothenic acid (53) (N-(α -hydroxy- β - β '-dimethylolbutyryl)- β -alanine) is also active biologically. On the other hand, when α -alanine, β -aminobutyric acid, lysine, or aspartic acid are coupled with the non-nitrogenous fraction of pantothenic acid, the resulting compounds are biologically inactive (54). It is to be recollected that β -alanine is also a constituent of anserine and carnosine. It is likewise of interest to point out again that β -alanine can be formed from aspartic acid by bacterial action (aporrhegma).

14. Epinephrine. According to Schuler and coworkers (55) the *in vivo* synthesis of epinephrine (adrenaline) proceeds as follows:

Devine (56) found that in the presence of surviving adrenal tissue, epinephrine was formed from phenylethylamine, tyramine, and phenylalanine, the first mentioned compound giving the highest yield. The possibility that adrenal tissue contains an unidentified catechol derivative (Catechol X) which may play a rôle in the conversion of tyrosine to epinephrine, exists. Epinephrine is formed when catechol x is incubated with surviving adrenal tissue. It is evident that no clear-cut mechanism of epinephrine synthesis has as yet been demonstrated.

15. Betaines. The close chemical relationship between the amino acids and the betaines suggests that the latter group of compounds exist in solution as dipolar ions (see page 243). The evidence for this statement is based on dielectric measurements by Devoto and coworkers (57) and Edsall and Wyman (58). The amount of the polar and the non-polar forms probably varies with different betaines. It is to be expected that if the positive charge of the dipolar betaine ion is located at or near the center of the nitrogen atom, the moment should be very close to that of the corresponding amino

acid. Hence the molar dielectric increment, $\frac{\Delta \epsilon}{\Delta c}$, of the two com-

pounds should be nearly the same. For glycine $\frac{\Delta \epsilon}{\Delta c}$ = 23; for its be-

taine
$$\frac{\Delta \epsilon}{\Delta c} = 18$$
; for δ -aminovaleric acid $\frac{\Delta \epsilon}{\Delta c} = 63$; for its betaine $\frac{\Delta \epsilon}{\Delta c}$

=60 (see page 884).

Nordt and Trieschmann (59) determined the approximate heats of sublimation of the betaines of p-aminobenzoic acid and of sulfanilic acid to be 24–44 and 12–20 Kg. Cal., respectively, indicating that these molecules are heteropolar. Reasoning again on the basis of similarity in structure between amino acids and betaines, it is to be expected that the latter compounds should, at least in part, exist as zwitterions even in the solid state. Better evidence based on experimentation to test this statement is desirable.

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CHAPTER VI

PEPTIDES, PEPTIDASES, AND DIKETOPIPERAZINES

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1. Peptides and Peptidases. Progress in this field has been amazingly rapid in the last few years, due principally to the work of Bergmann in utilizing a wide variety of new synthetic peptides for the characterization of the proteolytic enzymes, of Johnson in clarifying the nature of the intestinal peptidases, and of du Vigneaud in the elucidation of the factors involved in the carnosine effect.

Since extended discussion of the last-mentioned work is outside the scope of this book, there need only be mentioned the peptides that du Vigneaud and his collaborators prepared for the study of this problem: β -alanyl-l-histidine (90), d- β -amino-n-butyryl-l-histidine (91), d- and l- β -aminoisobutyryl-l-histidine (91), γ -aminobutyryl-l-histidine (92), glycyl-l-histidine (92), α -d- and α -l-alanyl-l-histidine (93), β -l-aspartyl-l-histidine (94), and β -alanyl-d-histidine (95).

Until recently it was assumed that the principal peptidases of intestinal erepsin were an aminopolypeptidase whose specific substrate was leucyldiglycine and a dipeptidase whose specific substrate was leucylglycine (96, 97, 98). Linderström-Lang (99, 100) however provided evidence that leucyl peptides of varying length were hydrolyzed by a single enzyme which he called leucylpeptidase. This discovery demonstrated the influence of the nature of the side chain on enzymic specificity. It was confirmed and extended by Johnson and his coworkers (101, 102, 103, 104, 105) who succeeded in separating the ereptic leucylpeptidase from an aminopolypeptidase. The latter's substrates were found to be tripeptides of the simple amino acids containing glycyl, alanyl, or prolyl as the acyl residue in the chain. A further distinction between the two enzymes lay in the fact that leucylpeptidase was activated by Mg and Mn ions whereas aminopolypeptidase was unaffected by the presence of these ions.

In order to avoid the titration procedures in the estimation of ereptic peptidase activity, Greenstein used the peptides of *l*-cystine

as substrates (106). The latter are quite soluble; when split by the peptidases, the insoluble cystine crystallizes out and can be separated and estimated. The physical chemical properties of the cystine peptides have been reported by Greenstein, Klemperer, and Wyman (107). Each half of the double molecules behaves independently because of the free rotation about the S-S bond.

The work of Bergmann and his collaborators on the characterization of the proteolytic enzymes ranks among the most masterly accomplishments in the field of biochemistry in this generation. Chapter VI of this book describes the numerous attempts to devise a peptide synthesis which would permit the preparation of a wide variety of synthetic substrates for the proteolytic enzymes. This particular phase culminated in the discovery of the carbobenzoxy method of Bergmann and Zervas (108) (page 262 of this volume). Armed with this method, which permitted the synthesis of any amino acid in whatever position desired in the peptide chain, exploration into the hitherto uncharted field of enzymic specificity could be attempted. In rapid succession Bergmann and his collaborators demonstrated (a) that enzymes hitherto believed to act only on proteins would split suitably arranged synthetic peptides of low molecular weight, (b) that the specificity of the proteolytic enzymes was not determined by the chain length of the substrate but by the nature of the latter's side chain and its relative position to the peptide bond hydrolyzed, (c) that the intracellular proteolytic enzyme referred to as cathepsin is a mixture of enzymes some of which have specificities quite similar to those found for enzymes in the gastrointestinal tract, (d) that peptides containing optically antipodal amino acids were split by different enzymes, (e) that with proper substrates the enzymic synthesis of peptides can be demonstrated under the same conditions employed for peptide hydrolyses by the same enzymes, and (f) that the process of protein digestion may consist of a sequence of coupled reactions involving several substrates and several enzymes, including synthetic as well as hydrolytic reaction steps.

No attempt can be made here to describe the very large number of new synthetic peptides prepared by Bergmann and his collaborators. Only those will be mentioned which illustrate the specificity of the particular enzymes described. Table I summarizes

¹ Reference to the large number of papers by these authors in the Journal of Biological Chemistry may be found in the recent review by Bergmann and Fruton (Advances in Enzymology, Nord and Werkman, 1, 63 (1941).

the specific substrates for each of the proteolytic enzymes studied by Bergmann, Fruton, et al., and is representative of the latters' recent classification of these catalysts (109).

Table I
Specificity of Proteolytic Enzymes (according to Bergmann)

Enzyme	Substrates*†	Requisite peptide chain	Requisite side group R	Cysteine activation
Pepsin Cathepsin I (spleen, kidney)	Cbzo-l- glutamyl- l- tyrosine Cbzo-l- glutamyl- l- phenylalanine	R —CONH·CH·CONH— ↓↑	HO CH:-	Pepsin—none Cathepsin I— none
Trypsin Cathepsin II (spleen, kidney) Papain	Benzoyl-l- lysine amide Benzoyl-l- arginine amide	R CONH·CH·COOH +NH:	NH ₂ (CH ₂),— or H ₂ N CNH(CH ₂) ₃ —	Trypsin—none Cathepsin II— positive Papain—positive
Chymotrypsin	l-Tyrosyl glycine l-Phenylalanyl glycine	R CONH- CH·CONH- ↑↑ R COOH+NH;CHCONH	HO CH -	Chymotrypsin —none
Leucylpeptidase (intestine) Cathepsin III (spleen, kidney)	l-Leucyl glycine	R NH:CH·CONH— ↓↑ R NH:CH·COOH+NH;	H ₂ C CHCH ₂ —	Leucylpeptidase —none Cathepsin III— positive
Carboxypeptidase (pancreas) Cathepsin IV (spleen, kidney)	Cbzoglycyl l-tyrosine Cbzoglycyl l-phenylalanine	R -CONH·CH·COOH ↓↑ R -COOH+NH ₂ ·CH·COOH	HO CH:-	Carboxypeptidase—none Cathepsin IV— positive

^{*} Cbzo = C₆H₅CH₂OCO(carbobenzoxy).

It is indeed remarkable that enzymes originating from different organs, such as the stomach and the kidney, should exhibit the same type of substrate specificity. The enzymes of the digestive tract and of the internal organs are, however, not at all identical in other respects. The pH optima of the former are quite different from those of the latter, and with the exception of cathepsin I, the intracellular enzymes act on their substrates in the presence of an

[†] Vertical dotted lines indicate position of hydrolysis.

activator such as cysteine. The identities of cathepsins I to IV in each of the tissues studied were distinguished by a study of the reaction velocities on a number of the synthetic substrates after treatment of the tissue preparations by various means such as addition of activators or inhibitors, dialysis, and heating. Bergmann has referred to enzymes that belong in a single group designated by related substrates with identical peptide chains, such as pepsin and cathepsin I, or carboxypeptidase and cathepsin IV, as homeospecific whereas enzymes belonging to different groups such as trypsin and chymotrypsin are called heterospecific. Studies of the reaction kinetics of each of the enzyme groups revealed that the ratios of the first order reaction constants for the various pairs of substrates in each group (Table I) were practically identical in each group and quite different from group to group (109).

To the substrates in Table I must be added the peptides of proline in which the imino group of the latter is bound in peptide linkage, thus — CO·N=. An enzyme that hydrolyzes this linkage has been found in the intestinal tract and has been termed prolidase (110). Crystalline synthetic substrates for cathepsin preparations from normal and from tumor tissues have been recently employed by Maver, Johnson, and Thompson (111). These investigators used leucyldiglycine, triglycine, leucinamide, glutamylanilide, hippuryl glycine, chloracetyltyrosine, glycyltyrosine, carbobenzoxyglutamyl-

glycine, glutamylglycine, and glutathione.

In a series of papers, Bergmann and his collaborators have demonstrated that peptides containing the d optical isomers of the amino acids can be hydrolyzed by the enzymes of papain, yeast, and the intestinal mucosa (112, 113, 114). Berger, Johnson, and Baumann (104) further showed that when the peptidases from chick mucosa, yeast autolysate, and malt were suitably activated with Mn, they hydrolyzed d-leucylglycine about one-thirtieth as rapidly as dl-leucylglycine. Smith and Bergmann (115) have shown that the intestinal enzyme which attacks l-leucylglycine is different from the enzyme which attacks d-leucylglycine.

2. Enzymic Peptide Synthesis. The enzymic hydrolysis of a peptide bond is theoretically reversible and the synthesis of such a bond should be demonstrable. The equilibrium of such a reaction, however, lies far on the side of hydrolysis and it is necessary to find conditions whereby the concentration of the synthetic product may be kept continuously below the equilibrium concentration. Bergmann and Fraenkel-Conrat first solved this problem by using hip-

puric acid and aniline in the presence of cysteine-activated papain (116). The very insoluble hippurylanilide crystallized out. Since then, Bergmann and his coworkers have succeeded in synthesizing a large number of peptides by means of several enzymes.² The conditions chosen, i.e., concentration of reactants and enzymes, pH,

TABLE II

Enzymatic Synthesis of Peptides (Bergmann)

Reactants*	Enzyme Products		Refer- ence	
Hippuric acid + Aniline	Papain	Hippurylanilide	(116)	
Cbzoglycine + Aniline	Papain	Cbzoglycylanilide	(116)	
Hippurylamide + Aniline	Papain	Hippurylanilide + Ammonia	(116)	
Cbzoglycine + Phenylhydrazine	Papain	Cbzoglycylphenylhydrazine	(116)	
Hippuric acid + Phenylhydra- zine	Papain	Hippurylphenylhydrazine	(116)	
Benzoyl-dl-leucine + Alinine	Papain	Benzoyl- <i>l</i> -leucylanilide +Benzoyl- <i>d</i> -leucine	(116)	
Benzoyl-l-phenylalanine + Aniline	Papain	Benzoyl- <i>l</i> -phenylalanylanil- ide	(116)	
Acetyl-l-phenylalanine + Phenylhydrazine	Papain	Acetyl- <i>l</i> -phenylalanylphenyl- hydrazine	(116)	
Benzoyl-l-alanine + Aniline	Papain	Benzoyl- <i>l</i> -alanylanilide	(116)	
Benzoyl-l-leucine + Aniline	Bromelin	Benzoyl-l-leucylanilide	(116)	
Benzoyl-l-leucine + Aniline	Cathepsin	Benzoyl-l-leucylanilide	(116)	
Acetyl-dl-phenylalanyl glycine + Aniline	Papain	Acetyl- <i>l</i> -phenylalanylglycyl anilide + Acetyl- <i>d</i> -phenyl- alanylglycine	(117)	
Benzoyl- <i>l</i> -leucine + <i>l</i> -leucylanilide	Papain	Benzoyl- <i>l</i> -leucyl- <i>l</i> -leucyl- anilide	(118)	
Benzoyl-l-leucine + glycylanilide	Papain	Benzoyl- <i>l</i> -leucylanilide +glycine	(118)	
Acetyl-l-phenylalanyl-l- glutamic acid + Aniline	Papain	Acetyl-l-phenylalanyl-l- glutamylanilide	(118)	
Benzoyl- <i>l</i> -tyrosine+ glycylanilide	Chymo- trypsin	Benzoyl- <i>l</i> -tyrosyl-glycyl- anilide	(119)	
Cbzo- <i>l</i> -phenylalanylglycine + <i>l</i> -tyrosine amide	Papain	Cbzo- <i>l</i> -phenylalanyl- glycyl- <i>l</i> -tyrosineamide	(120)	

^{*} Cbzo = $C_6H_5CH_2OCO$ —(Carbobenzoxy).

temperature, and activators were the same as those used in hydrolytic experiments. Table II lists the peptides synthesized.

The reactions described in Table II include the resolution of racemic amino acids by asymmetric enzymic synthesis (117) (121) and what appears to be a coupled hydrolysis and synthesis as in the

² By the use of the kidney tissue slice technique, Borsook and Dubnoff (125) demonstrated the *in vitro* synthesis of hippuric acid.

case of the reaction mixture of hippurylamide, aniline, and papain (116). In the latter case one of the reactants, hippurvlamide, is not only soluble but it is also readily susceptible to papain hydrolysis. It is therefore split into hippuric acid and ammonia, and the former combining with aniline in the presence of the same enzyme forms the insoluble anilide which precipitates from the solution. However, other kinds of soluble reactants, such as glycylanilide or glycyl-leucine, which are completely resistant to papain, will be hydrolyzed by this enzyme when other soluble peptides are present. Thus Behrens and Bergmann (120) found that glycylanilide is hydrolyzed to glycine and aniline in the presence of horse serum and papain, and that glycylleucine yields glycine and leucine in the presence of acetyl-l-phenylalanylglycine and papain. In the latter case Behrens and Bergmann postulate a synthesis of the two peptides to form acetyl-l-phenylalanylglycylglycylleucine from which leucine and then glycine are successively hydrolyzed. The overall reaction, the hydrolysis of the otherwise resistant glycylleucine, is thus the sum of three coupled reaction steps, leaving the acetylphenylalanylglycine untouched at the end. Substances like the latter which perform an obviously catalytic rôle have been termed "cosubstrates" by Behrens and Bergmann. The hydrolysis of glycylanilide in the presence of serum or casein and papain is similarly due to the providing by these partially digested proteins of peptides which function as cosubstrates toward the otherwise resistant glycylanilide. These remarkable experiments provide a new concept of the process of protein digestion for they suggest that proteolysis may consist not of a simple, single step degradation of the protein but rather of a large number of complex, concurrent and coupled hydrolyses and syntheses which ultimately yield the mixture of amino acids. At any one time in the course of the digestion, therefore, the composition of the polypeptides in the reaction mixture will not necessarily represent the order of amino acids present in the peptide chains of the intact protein before digestion. If the reverse of this complex chain of events is considered, namely, the biological synthesis of proteins, it is little wonder that the latter compounds possess an almost limitless diversity.

³ Ornithine in the Krebs cycle behaves in the fashion of a cosubstrate for it couples with carbon dioxide and ammonia to form arginine which is hydrolyzed to urea and ornithine. The latter, like acetylphenylalanylglycine, is regenerated. While the function of ornithine is to produce a synthetic product of NH₃ and CO₂, and that of acetylphenylalanylglycine is to cause the hydrolysis of another peptide, both accomplish these aims by first combining with the respective reactants.

3. The Diketopiperazines. Complex representatives of this class of substances have been prepared by du Vigneaud, Patterson, and Hunt (122) and by Greenstein (123). The latter prepared the crystalline diketopiperazine of *l*-cysteine, the former group the crystalline diketopiperazine of *l*-homocysteine. Each molecule possesses two sulfhydryl groups and on controlled oxidation should yield a compound of the disulfide form. It was found that the former on oxidation yielded a soluble crystalline dimer of cystine diketopiperazine whereas the latter yielded an insoluble polymer of high molecular weight of the diketopiperazine of homocystine.⁴

⁴ The diketopiperazine of l-cystine has been studied in Dr. Astbury's laboratory by Miss Bell. The latter described (124) the crystals as monoclinic, the unit cell having the dimensions a, 11.15 Å; b, 5.9 Å; c, 12.5 Å; and $\beta = 90^{\circ}$. The number of molecules per unit cell was 2. Preliminary calculations revealed that the rings are about 6.25 Å units apart, and are probably not flat but puckered into the chair form. The double ring molecule behaves optically almost exactly like two separate molecules related to each other by a dyad screw axis.

Treatment of the diketopiperazine of cysteine with strong HCl vielded l-cysteinyl-l-cysteine (123). Oxidation of the latter produced the crystalline dimer l-cystinyl-l-cystine. Studies of the behavior of model substances of the kind described are of value in protein chemistry since the possibility exists of oxidation of the numerous thiol groups in certain proteins under physiological conditions. Whereas the behavior on oxidation of molecules containing a single mercaptan group has long been known, little or no information has been available on the comparable behavior of polythiol molecules.

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CHAPTER VII

THE CHEMICAL CONSTITUTION OF THE PROTEINS

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In this Addendum, an attempt will be made to comment, modify, and bring up to date the statements made five years ago.

- 1. Classification (pp. 278-9). Little progress has been made in the chemical classification of proteins. The inadequacies of solubility as the basis, especially for albumins and globulins, are now generally recognized. However, the keratins have been divided into two classes (167, 168). The first or eukeratins have been defined earlier (p. 279) under the heading Keratins; the second more heterogenous group have been called pseudokeratins. They are insoluble, enzymic-resistant proteins, which yield lysine and arginine in the molecular ratio of approximately 4:6 (167).
- 2. Dehydration of Protein Solutions (p. 280). The technique for drying blood serum and other protein solutions in the frozen state has been strikingly advanced during the past few years. One of the special kinds of apparatus used has been described by Flosdorf, Stokes, and Mudd (169).
- 3. Isolation of Proteins (p. 282). The isolation of proteins, especially from bacteria, by the use of supersonic vibration or prolonged grinding to break down the cell structure has proved useful. Also, considerable use of proteolytic enzymes has been made recently by bacteriologists and immunologists to separate and purify specific proteins. It may be that certain proteins, usually of a fibrous structure,—keratins, myosin, antibodies (170), tobacco mosaic virus, etc.—are, in their native state, more resistant to enzymic digestion than are the more globular proteins. However, the value of enzymes for the separation of proteins has long been known (171).

Banga and Szent-Györgyi (172) have suggested a solution of 30 per cent urea with either KCl—Na₂CO₃—NaHCO₃ buffer or with 2 per cent NaOH as a solvent for the more insoluble, fibrous structural proteins.

4. Hydroxyglutamic Acid (pp. 285-6). The failure of many com-

petent investigators to obtain appreciable quantities of β -hydroxy-glutamic acid from protein hydrolysates may be taken as justifiable evidence that this amino acid is not among the naturally occurring hydrolytic products of proteins. Previous reports of the presence of this amino acid in the dicarboxylic amino acid fraction may have been due to serine as an impurity (173) or possibly to γ -hydroxyglutamic acid (174).

- 5. Sulfur (p. 287). The investigations of Rose, du Vigneaud, Schoenheimer, Borsook, and others have done much to elucidate our ideas of the importance of methionine (cf. (175)). Methionine, on enzymic fission, liberates a methyl group which can react with guanidinoacetic acid (from glycine and arginine) to form creatine. It also appears that methionine and choline are in equilibrium with respect to their methyl groups. Thus methionine will split to yield homocysteine and a methyl radical and the latter can then unite with aminoethanol; or choline will yield methyl groups capable of reacting with homocysteine (cf. (175)) to yield methionine. The interesting observation that the feeding of cystine increases the pathological changes in the liver and kidneys caused by a deficiency of a source of methyl groups (methionine or choline) awaits further adequate explanation (cf. (176)).
- 5a. Selenium. Horn and Jones (177) have isolated a crystalline amino acid complex from a weed growing on seleniferous soil which appears to be the mixed ether of cysteine and homocysteine. The substance isolated was an isomorphous compound containing Se and S in the ratio of 2 to 1. The suggested formula is: HOOC·CHNH₂·CH₂·Se·CH₂·CH₂·CH₂·CHNH₂·COOH where S can replace Se.
- 6. Nucleoproteins (pp. 290–2). Evidence has been accumulating that leads one to question whether the attachment of nucleic acids to proteins as suggested by Osborne and Campbell is valid. Sevag and Smolens (178) define nucleoprotein as a compound in which the protein and nucleic acid are combined by non-polar linkages and which, in an electrophoretic cell, migrates as a single entity. They call the products studied by Osborne and Campbell, Jones, and others protein nucleates. These are compounds of protein and nucleic acid combined by linkages of the polar type. When placed in an electrophoretic cell, the protein and the prosthetic components migrate separately. A simple test is given to differentiate these two groups. Protein nucleates will precipitate with neutral CaCl₂ while nucleoproteins do not. Astbury has suggested that some virus pro-

teins are nucleoproteins with each nucleotide coordinating with 54 amino acid residues (179).

Wrinch's assumption (cf. pp. 291–2) that chromosomes are bundles of polypeptide chains held together by transversely disposed nucleic acid molecules is not reconcilable with the observed birefringence of chromosomes and of nucleic acid. It seems that the molecules of nucleic acid in the chromosomes probably lie parallel to the polypeptide chains (180).

- 7. Vitamins as Prosthetic Groups (pp. 290-1). One of the most interesting developments of recent years has been the finding that some of the vitamins of the B complex function as prosthetic groups for specific proteins. Thus, thiamine-pyrophosphate is coniugated with a protein to form carboxylase (181). The dinucleotides composed of adenine, nicotinic acid amide, two molecules of ribose, and two or three molecules of phosphoric acid (diphosphopyridine nucleotide or triphospopyridine nucleotide) unite with various proteins to function in the oxidation of numerous carbohydrates (cf. (182)). A third member of the vitamin B complex, riboflavine as flavine-adenine-dinucleotide, when joined to specific proteins, forms d-amino oxidase, xanthine oxidase, etc. It has been shown that the protein component is the only one in the oxidation enzyme system that possesses specificity as it is the substance that activates the oxidizable substrate. Thus one prosthetic group may oxidize a diversity of substrates when it is combined with various specific activating proteins and, on the other hand, one prosthetic group may be substituted by another and yet the enzyme system will function satisfactorily.
- 8. Metalloproteins (pp. 293-6). (a) Iron. An extensive investigation of the respiratory enzyme, cytochrome c has shown that the protein component of this substance is unusually rich in lysine (15 to 25 per cent) but yields only a little over 3 per cent of histidine and less than 3 per cent of arginine (183). This contrasts with horse hemoglobin which yields 8 per cent of lysine, 7.5 per cent of histidine, and over 3 per cent of arginine (184). Cytochrome c has a molecular weight of approximately 13,000. The hemin molecule is presumed to be built into the protein by means of the thioether linkages resulting from the saturation of the double bonds of the vinyl groups of the porphyrin with the—SH of cysteine and by a salt (?) linkage between Fe and two imidazole rings of histidine (183). Theorell and Åkesson (183) believe that the hemin fits into a crevice of the protein molecule in such a fashion that neither O₂,

- CO, or HCN can approach the Fe atom. This, they believe, explains why cytochrome c is not autoxidizable or why the carbon monoxide and cyanide compounds are not formed at physiological pH ranges. Catalase, peroxidase, and uricase seem to be iron-protein-porphyrin complexes (cf. 182).
- (b) Copper. The importance of Cu in the nutrition of plants and animals has been known for some time but with the exception of its presence as a respiratory protein in certain invertebrates (cf. p. 294), its mode of action remained unknown until Kubowitz (19) found that polyphenol oxidase is a copper-protein complex. Laccase, tyrosinase, ascorbic acid oxidase, and other oxidation enzymes are Cu-protein complexs (cf. (182)).
- (c) Magnesium (p. 295). The eprotein complex for the decarboxylation of α -keto monocarboxylic acids (carboxylase) is said (181) to be a magnesium-diphosphothiamine-protein complex. The elements are combined in the ratio of 1:1:1.
- (d) Manganese (p. 295). It has been reported that arginase may require Mn or possibly Co as an essential metallic constituent (186).
- (e) Zinc (pp. 295-6). Like Cu, the nutritive importance of zinc for the maintenance of life in plants and animals was known before a definite physiological function was found for this metal. Carbonic anhydrase, found by Meldrum and Roughton in 1933, has been shown to be a zinc-protein complex containing either 0.15 (187) or 0.33 per cent of Zn (cf. (182)). This enzyme is specifically inhibited by sulfanilamide in concentrations as low as 2×10^{-6} M. As carbonic anhydrase is the enzyme concerned with the release of CO₂, the effects of sulfanilamide on respiration, long after administration of the drug, is explained. This effect was prominently brought out when it was found to be the cause of airplane crashes in the R.A.F. The pilots had received sulfanilamide and were discharged as cured as long as three weeks before their fatal flights.

It is to be expected that other elements, required in traces for life processes, will be found linked to proteins as the prosthetic group of enzyme systems.

9. Glycoproteins (pp. 296-7). Proteins unite with carbohydrates in two ways. The first, by polar linkages, is with carbohydrates which contain "free" carboxyl or sulfuric acid groups. The second, more stable form, is with neutral polysaccharides to form true glycoproteins (188). Przylęcki (189) believes that carbohydrates can combine with soluble proteins via the imidazole group of histidine to form glycoproteins (cf. p. 284). Thyroglobulin, crystalline serum albumin, and pregnancy urine hormone are considered to be

glycoproteins. It is of interest that the presence of any prosthetic grouping appears to protect the protein moiety against denaturation. A convenient way to prepare many conjugated proteins, especially those in which the prosthetic component comprises a large proportion of the molecular complex, is to remove the soluble contaminating proteins by shaking an aqueous or saline solution with chloroform or chloroform and amyl alcohol. The denatured globular proteins are then readily removed. This procedure has been successfully employed for the preparation of ovomucoid, serum mucoid, bacterial nucleoprotein, hemocuprein, etc.

A complex containing polysaccharide, phospholipin, and polypeptide has been isolated from bacteria (190).

- 10. Halogenated Proteins. On page 299, we wrote "at present, no physiologically active iodized protein has been prepared." However, in 1936, a preliminary note by Ludwig and von Mutzenbecher appeared (191) claiming the synthesis of thyroxine when slightly alkaline solutions of iodine were allowed to act on proteins (casein, silk fibroin, edestin, and seralbumin) or on diiodotyrosine. These results have been confirmed by Harington (192) and by Block (193). Thus it appears that thyroxine can be synthesized by the coupling of two molecules of diiodotyrosine with the loss of one side chain. This unique reaction demonstrates again that amino acids are an unusual group of compounds. Their distinctive behavior is greatly exaggerated when amino acids unite to form a protein and this undoubtedly accounts for the many and diverse specific and unique features of proteins.
- 11. Hypotheses of the Structure of Proteins² (pp. 299–329). It may be well, before discussing the various protein hypotheses, to define the term hypothesis. Hypothesis refers to "something not proved but assumed for the purpose of argument. A system imagined or assumed to account for what is not understood." Realizing the meaning of hypothesis, it is no reflection on the original proponent, as some critics assume, when further experimental evidence proves a currently acceptable hypothesis to be no longer tenable. It is in the nature of science that an hypothesis is but a guidepost toward the ultimate goal.

¹ The mechanism of the reaction has been explained by Johnson, T. B. and Tewkesbury, L. B., *Proc. Natl. Acad. Sci. U. S.*, 28, 73 (1942).

² In this discussion, it is taken for granted that the general theories of polypeptide structure promulgated by Hofmeister and by Fischer (cf. pp. 301-3) are valid. However, Linderstrøm-Lang (231) has recently presented some evidence that this may not be the case in all proteins.

- (a) Bergmann's Periodicity Hypothesis¹ (pp. 304–5). Bull (194) has pointed out that 288 has the largest number of exact divisions of any number between 0 and 576. By assuming a total number of amino acid residues of 288 rather than some other number, less violence is done to the analytical values in order to make them fit the theory. Some data in support of the stimulating periodicity hypothesis of Bergmann and Niemann has been given previously (pp. 304–5); however, experiments against this idea have been summarized by Bull (194) and others as follows:
- 1. There is no evidence from x-ray studies of a regular and invariant periodicity.
- 2. Calculation of the average residue weight is uncertain. If the average residue weight is in error, the calculated frequencies will be incorrect.
- 3. It is very doubtful if the present methods of amino acid analyses yield results for proteins that are sufficiently accurate to be used in the manner in which Bergmann uses them. The vast majority of amino acid analyses can, at present, be used for comparative purposes but absolute values have seldom been determined. The methods that now yield results suitable for comparative purposes are being constantly refined with the ultimate purpose, of course, of determining absolute values. Neuberger (195) has pointed out that the average error in amino acid methods is so great as to indicate with a very high degree of probability that a purely random distribution of amino acids would give values in apparent accordance with the formulae of the frequency hypothesis.
- 4. Bergmann and Stein (196) found that the quantities of glycine and proline in gelatin lead to a frequency of 3 for glycine and of 7 for proline. Thus, there would be a conflict between these two amino acids for every 21st position, if the two amino acids are to have invariant periodicities in the polypeptide chain.
- 5. The number of amino acid residues calculated from physical data and analytical results for lactalbumin, egg albumin, insulin, horse hemoglobin, and edestin do not fit the number of residues demanded by the Bergmann hypothesis (194).
- 6. Estimation of the average residue weight by titration for lactoglobulin (197) and for insulin (198) indicates 116.7 for the former and 123.2 for the latter. If the molecular weight of these proteins as reported by Svedberg is taken as correct, then it is found that lactoglobulin contains 349 and insulin 332 amino acid residues per molecule.

¹ See Chibnall, A. C., Proc. Roy. Soc., 131 B, 136 (1942).

- 7. Further evidence against Bergmann's hypothesis is that degredation of proteins to enzymic-resistant peptones and peptides often results in products containing a disproportionately large concentration of one or two amino acids (cf. (173)). On the other hand, very mild digestion (5 minutes at pH 2.1) of horse serum albumin with pepsin (0.025 per cent) yields an undialyzable product approximately $\frac{1}{4}$ the size of serum albumin. Although less than 10 per cent of the protein nitrogen became dialyzable by this procedure, the dialysate contained over 90 per cent of the tryptophane of the original protein (199).
- (b) Proteins as Reversibly Dissociable Component Systems (Sørensen) (pp. 309-312). With the more widespread use of the Tiselius electrophoretic methods and various modifications of Svedberg's ultracentrifuge, doubt (cf. (194)) has been cast on the hypothesis that soluble proteins are composed of reversibly dissociable component systems as expressed by Sørensen and others. It appears as generally acceptable that the classification of serum proteins as albumins and globulins by simple salting-out methods is no longer tenable (200). However, it is extremely difficult to correlate the physical investigations which study the protein complex of serum in its "native state" with the results of workers who isolate proteins by chemical means. The uncertainty is increased by the finding that some proteins such as recrystallized egg albumin which were believed to be homogeneous, are now found to contain two components (201). In fact, both ultracentrifuge and electrophoretic measurements may fail to show inhomogeneities. The situation is further complicated by the finding that various substances, especially proteins, in solution affect the molecular size (degree of dispersion) of proteins. This interaction of one protein in solution with another has led Pedersen (202) to suggest that proteins have a primary and a secondary structure. The primary structure is the polypeptide chain which is probably arranged in some kind of definite unit. These units are linked together by secondary forces which may be either proteins, polypeptides, or other substances. Many substances may be adsorbed on the native protein without having anything to do with the structure of the molecule. The strength of the linkages between the components of a protein complex varies markedly. The great tensile strength of the natural polymers is, in general, due to their chain molecular structure. In the case of cellu-

³ It is obviously impossible to study serum in its "native state" as serum does not exist until the blood has been drawn from the circulatory system.

lose the strength is comparable to that of the finest steel (cf. (180)). It may be assumed that the polypeptide chain in proteins is also remarkably strong, viz., silk fibroin. On the other hand, studies with the ultracentrifuge (202) have shown numerous instances of proteins dissociating into halves, thirds, quarters, etc. These fractions have been found to be of dissimilar chemical composition. [It may be that such proteins are molecular compounds rather than molecules (203).] By changing the conditions, the molecular fractions have been made to recombine so as to yield what appears to be, but probably is not, the original molecule. Although reversibly dissociating molecules may be held together by relatively weak forces, if the molecule is essentially one unit, dissociation cannot occur without the disruption of at least one linkage. This may lead to a loss of the intrinsic structure, i.e., denaturation, unless the native state of the protein is its most stable configuration.

The interconnection of certain soluble proteins with each other and the non-protein constituents of the tissue has been designated as the "orosin hypothesis." The concept was used to denote the interrelationship among the various proteins found in plasma or serum by physical and chemical means. The evidence in favor of this hypothesis was based on the relative constancy in basic amino acid composition in cases where the albumin-globulin ratio changes. Confirmatory evidence for this idea has been found by Murrill and Newburgh (204) for arginine, histidine, and lysine, and by Bálint (205) for tyrosine, tryptophane, and cystine. However, under certain pathological conditions (multiple myeloma (206), nephritis (207)), there is a change in the amino acid composition of orosin. At the present time, the question whether plasma contains a small number of distinct proteins that are in every respect chemical individuals and in which the difficulties of isolation are due to a lack of specificity of the analytical methods used; or whether the proteins of plasma contain an indefinite number of molecular species that fall into groups of certain physical and chemical similarity—a fact that would be responsible for an apparent and deceptive uniformity in physical and biological behavior of different fractions, is unsettled.4 However, current opinion favors the first hypothesis.

(c) The Basic Amino Acid "Anlage" Hypothesis (Block) (pp.

⁴ In discussing the proteins of egg white, Young (232) says "The results suggest that we are creating artifacts in our efforts at separation by means of various solvents. The natural protein of egg white may be a mucoprotein-albumin complex broken by the tools which we use to separate it."

312-14). This hypothesis has been criticized (208) on the basis that variations in the experimental ratios of histidine to lysine to arginine are greater than would be normally ascribed to an experimental error; in general, the constancy of the ratios that would be expected if the "Anlage" hypothesis were correct is apparently not observed. On the other hand, experimental support for the hypothesis that the basic amino acids may be of unique importance in the

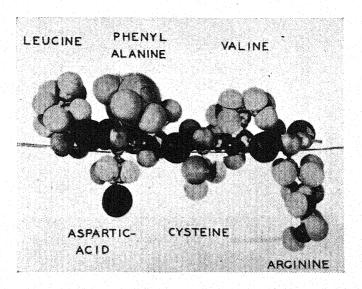


Fig. 1. Model of a Peptide. (Neurath, H., J. Phys. Chem., 44, 296 (1940).)

development of the eukeratins has appeared (168). However, there is little evidence that these three amino acids are of special importance in the development or structure of proteins other than the limited group of eukeratins.

(d) Molecular Weights of Proteins (Svedberg Unit) (pp. 314-5). More recent estimations of the molecular weight of proteins by both ultracentrifuge and diffusion methods make it appear likely that the so-called Svedberg unit is a matter of coincidence (209). The multiple law cannot be regarded as sufficiently established to be used in support of any theory of protein structure. Proteins with molecular weights of 10,000, 13,000, 26,000, 40,000, etc., have been found in sufficient number to throw doubt on the unit concept. Bull's (194) analysis of Svedberg's own data also supports the conclusion that this interesting idea may have to be discarded.

(e) X-ray Studies (Astbury) (pp. 315–25). The general ideas concerning the β -keratin chain are largely accepted (Fig. 1) but the experiments of Neurath (210) have shown rather definitely that Astbury's configurations for α -keratin are much too condensed to permit residues other than glycine and possibly alanine to occupy

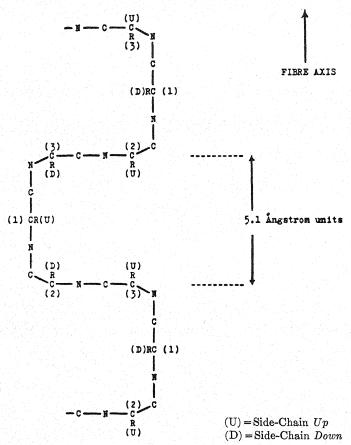


Fig. 2. Proposed Basis of Intramolecular Fold in α-Keratin and α-Myosin. (Astbury,
 W. T., Jubilee Memorial Lecture, Chemistry and Industry, 60, 491 (1941).)

the positions forced by the valence angles and bond distances of the carbon atoms of the hexagonal rings. As soon as any peptide chain is folded, all atoms attached directly to atoms in the peptide chain lose their freedom and the R-groups, instead of alternating above and below the chain as they can do in a β -keratin chain, (Fig. 1) would have to be all on the top or bottom of the chains where there is insufficient room to accommodate them. To circum-

vent this difficulty, Astbury has recently (211) modified his picture of the α -keratin linkage to give a more open chain (Fig. 2).

Although many interesting ideas concerning the fiber structure of proteins have come from x-ray analysis, it must be borne in mind that direct analysis of the x-ray photographs is rendered imimpossible by the fact that we can never know the phases of the reflections corresponding to the different spots (203). Astbury's

concept of intermolecular folding and stretching of hair is fundamentally different from the interpretation of highly reversible extensibility advanced by Meyer and Mark in the case of other polymers (212). Fig. 3 summarizes Mark's concept illustrating stretching without any change in internal energy. The chains are simply straightened (Fig. 3; b-a). The internal energy may even diminish on stretching. if, as a result of orientation, the chains are brought into alignment so that they form crystallites with the liberation of heat of crystallization. The stretched polypeptide chain (β -keratin) then becomes the least probable and most highly organized configuration.

The geometric organization⁵ and not the chemical nature alone of "living substances" may explain many of their distinctive properties. Thus, the insoluble silk thread is formed from the water solu-

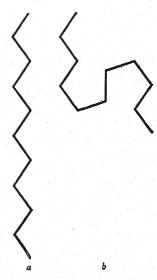


Fig 3. Schematic representation of stretching of a protein fiber without any change in internal energy. (Picken, L. E. R., *Biol. Rev.*, 15, 133 (1940).)

ble proteins of the silk glands of the caterpillar by stretching (213). It appears that the silk gland contains the proteins as a supersaturated solution which state is abolished by mechanical means, i.e., extrusion through the orifice of the gland. If the fiber structure is destroyed by grinding, the protein becomes soluble and no longer resistant to digestion by proteolytic enzymes. Other physical agents, such as ultraviolet light, acting through the phenolic group of tyrosine, also result in changes that disrupt the peptide chain

⁵ Certain fundamental differences in reaction of living and non-living systems were clearly brought out by Beams and associates (233). These investigators found that living protoplasm is capable of withstanding very great centrifugal forces without the separation of its essential elements, differing, in this respect, from many non-living colloidal systems.

- (214). The importance of physical structure on the chemical and physical properties has been mentioned on page 289. Recent investigations have confirmed and extended these findings (215, 216, 217) but even as long ago as 1877, Kühne (218) showed that very finely divided hair would be digested by pepsin. It seems to the writer that the grosser, more easily recognized properties of proteins [texture (167, 168), color (p. 293), resistance to enzymes (167, 168)] may be closely linked to the geometric structure of the molecule; while the finer, individual differences [immunological properties (page 293), specific quantitative biological activity (219), solubility (220)] such as are seen in an homologous series of proteins, are principally due to changes in the quantities of one or more amino acid residues.
- (f) The Cyclol Hypothesis (Wrinch) (pp. 395-9). This hypothesis, which has received widespread publicity, was based on little experimental evidence (cf. p. 327) and has been largely abandoned (194, 195, etc.). Some of the suggestions concerning the manner in which polypeptide chains are joined to each other have been given earlier in Chapter VII. However, the many specific models proposed by Wrinch and more recently by Huggins (221) to illustrate the structure of proteins are still little more than mere "paper formulae."
- (g) The State of Proteins in the Living Organism (Schoenheimer). The experiments of Schoenheimer and his associates (222) have revealed that in the living organism there is no such thing as a permanent protein molecule but that proteins are being constantly broken down into amino acids and newly built up again. The protein and its components in the metabolic mixture are, therefore, in a continuous state of exchange.
- (h) d-Amino Acids in Proteins. Jacobs and Craig (223) obtained d-proline from ergot alkaloids and Ivánovics and Bruckner (224) found d-glutamic acid as the principal constituent of the capsule of Bacillus anthracis and other microorganisms of the subtilismesentericus group. The resistance to enzymic digestion of these polypeptides or proteins, composed largely of d-glutamic acid, led Kögl and Erxleben (225) to look for unnatural amino acids in cancer tissue. They reported the occurrence of the unnatural isomers of several amino acids and especially of d-glutamic acid. However, their hypothesis of cancer formation had to be abandoned when Chibnall (226) and others found that the quantities of d-glutamic acid isolated by Kögl and Erxleben were not more than could be

obtained from normal plant and animal proteins and were probably due to racemization during hydrolysis.

The occurrence of enzymic resistant bacterial polypeptides containing d-amino acids has been demonstrated in the culture medium of the soil organism, Bacillus brevis (227, 228). Hotchkiss and Dubos (229) have isolated two polypeptides that are highly toxic for Gram-positive microorganisms and also to animals when injected into the blood stream. One of the substances, gramicidin, yielded 35 or 40 per cent l-tryptophane (228, 230), 22 per cent d-leucine (228), 23–33 per cent of alanine (228), and 0.0 per cent of tyrosine (230). Another bacteriocidal polypeptide, tyrocidine, yielded 13.3 per cent of tyrosine (230), and 5–6 per cent of tryptophane according to Christensen et al. (230) or 16–17 per cent according to Hotchkiss (228).

Summary (p. 328). The general principles given under this heading 5 years ago should be modified as follows. 2. The basic amino acids may plan an especially important rôle in the genetic development of the eukeratins. 4. Evidence is beginning to accumulate against the concept of a "protein unit" of 17,600 or some simple multiple thereof.

Conclusion (pp. 328-9). The past five years have seen the gradual abandoning of the majority of protein hypotheses that looked so promising in 1937 and 1938. Some of these have disappeared entirely, others are being modified as new experimental data appear or the old are reinterpreted, and finally new hypotheses, more firmly based on experimental fact, are being advanced.

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CHAPTER VIII

MOLECULAR WEIGHTS OF THE PROTEINS

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Information on the molecular size of the proteins continues to accumulate at a rapid rate. Most of the new data have been obtained by means of the ultracentrifuge. A detailed description of this instrument and its applications has been published in book form by Svedberg and Pedersen (103). Additional data on the molecular weights of the proteins obtained by the ultracentrifuge are given in Table I. Certain heretofore almost unused physical principles have been applied to the problem of the molecular size of the proteins. These include viscosity, dispersion of the dielectric

Table I

Molecular Weights and Molecular Constants of Proteins as Determined
by the Ultracentrifuge¹

Protein	Source	Isoelect. Point	S20×1013	D20×107	Mv	M_E	f/fo
Lactalbumin	Cows' Milk	5.12	1.9	10.6	17,400		1.1
Cytochrome C	Beef heart	9.7	1.9	10.1	15,600		1.2
Myoglobin	Muscle	7.0	2.0	11.3	16,900	17,500	1.1
Gliadin	Wheat		2.1	6.7	27,500	27,000	1.6
Hordein	Rye		2.0	6.5	27,500		1.6
Zein	Corn		1.9	4.0	40,000		2.4
Concanavalin B	Jack Bean		3.5	7.4	42,000		1.2
Crotoxin	Rattlesnake Venom	_	3.1	8.6	30,000	30,500	1.2
Yellow enzyme	Yeast	5.22	5.7	6.3	82,000	78,000	1.1
Concanavalin A	Jack Bean	_	6.0	5.6	96,000		1.2
Canavalin	Jack Bean	-	6.4	5.1	113,000		1.3
Antipneumococcus							
serum globulin	Man	-	7.4	3.6	195,000		1.5
Catalase	Beef Liver		11.3	4.1	250,000		1,2
Urease	Jack Bean		18.6	3.4	480,000		1.1
Bushy stunt virus	Tomato	4.11	146		7,600,000		1.0
Mosaic virus	Tobacco		200	0.3	60,000,000		3.0
Thymus nucleohistone ²	Calf	-	31	0.93	2,300,000	2,000,000	2.5

¹ This table is supplementary to Table XIV of Chapter VIII. With exception noted, data are taken from Svedberg and Pedersen (103). M_{2} =molecular weight by sedimentation velocity, M_{E} =molecular weight by sedimentation equilibrium, S_{20} =sedimentation constant at 20°, D_{20} =diffusion coefficient at 20°, f/f_{2} =dissymmetry number.

² From Carter, R. O., J. Amer. Chem. Soc. 63, 1960 (1941).

constant, double refraction of flow, and estimation of the unit cell by x-ray spectrograms.

1. Minimal Molecular Weight from Analytical Composition. The methods of determining the amino acid content of proteins have been improved considerably in recent years. When applied to homogeneous and well defined protein preparations, as is increasingly the case, data are obtained that may be employed in calculating the weight of the protein which contains one gram-atom or molecule of the particular component. This contribution to knowledge of the molecular weights of the proteins has been surveyed by Cohn (104) and by Vickery (105) in recent reviews. Important contributions to this field by the development of new reagents and a new principle for the analysis of the amino acid composition of proteins have been made by Bergmann and coworkers (106-108). The new principle consists of using the solubility product relation of an appreciable soluble amino acid salt to calculate the content of the amino acid in a hydrolysate rather than to determine the amino acid quantitatively.

Vickery (105) has examined the now existing methods for the estimation of the different amino acids and concludes that the procedures at least for cystine, tyrosine, tryptophane, methionine, aspartic acid, glutamic acid, arginine, histidine, and lysine appear to give satisfactory results after being widely tested. The methods now available for the other amino acids either have not received sufficient scrutiny or are frankly only of qualitative value.

One of the fundamental difficulties in accepting the analysis of the amino acid content of a protein is that there is no available method of hydrolyzing a protein that may not involve destruction of some of the amino acids.¹

2. Osmotic Pressure. The osmotic pressure method yields the average molecular weight under suitable experimental conditions (see Chap. VIII, page 348). In recent years this method for estimating the molecular weights of proteins has been used very little.² One reason for this is that the method is not sufficiently sensitive to cope with the measurement of the molecular size of such giant molecules as the virus proteins.

Since the first printing of this volume, Burk (109) has reported

¹ Steinhardt, J., and Fugitt, C. H., J. Res. Nat. Bur. Standards, 29, 315 (1942) hydrolyze proteins at 65° by means of low concentrations of cetylsulfonate or diphenylbenzenesulfonate ions. It has not yet been shown that complete hydrolysis may be so effected.

² Bourdillon, J., J. Biol. Chem., 127, 617 (1939) has described an apparatus for the determination of low osmotic pressures.

that the molecular weight of *Limulus* hemocyanin from osmotic pressure measurement in aqueous salt solution is 565,000, and in isoelectric urea solution it is 142,000.

Li, Lyons, and Evans (110) found the pure lactogenic hormone of the anterior portion of the pituitary gland from both the ox and the sheep to have the same molecular weight, namely, 26,500. Urea was found to have no dissociating effect on this protein hormone. The agreement in molecular weight in the hormone proteins from the two species is of interest since they differ in solubility, the hormone from the sheep being nearly twice as soluble as the analogous hormone obtained from the ox.

To check upon the revision of the molecular weight of crystalline egg albumin as determined by sedimentation velocity in the Svedberg ultracentrifuge (103) from 34,500 to 44,000, Bull (111) reinvestigated the osmotic pressure of this protein. The molecular weight value he obtained was 45,160 which agrees fairly well with the new results obtained by means of the ultracentrifuge.

Duclaux and Dobry (112) have found it possible to employ colloidion membranes to measure the osmotic pressure of proteins in non-aqueous solvents such as glacial acetic and lactic acids. With gliadin, the measurements indicated a molecular weight of about 40,000 in the above solvents and also in saturated magnesium perchlorate solution.

3. The Ultracentrifuge. The developments in Svedberg's oil-turbine drive ultracentrifuge since the first printing of this volume have been minor. Of outstanding importance have been the improvements in the air-turbine driven type of ultracentrifuge. The air-driven ultracentrifuge promises to have a widespread usage because of its comparatively low cost.

The cone-shaped air driven rotor was introduced by Henriot and Huguenard (113). But Beams and Pickels¹ (114) were the first to develop an air-turbine drive by which it is possible to obtain high rotational speeds with large rotors without exposing them to appreciable air friction. This was accomplished by attaching a length of straightened piano wire to a small cone-shaped air driven rotor of the Henriot and Huguenard type. The piano wire was extended downward through an oil gland into a vacuum chamber and then fastened to a large rotor. This wire served as a drive shaft. When a high vacuum was maintained in the chamber housing of the rotor, only a relatively small amount of driving energy was necessary to attain rotational speeds that were limited only by

¹ See also review by Pickels, E. G., Chem. Rev., 30, 341 (1942).

the strength of the material used for the rotor. It has been found most satisfactory to machine the rotor from aluminum alloy because of its low density and superior malleable properties. The rotors are made oval shape and the maximum speed obtainable is materially increased if the excess of metal is removed from the solid sections along the periphery farthest from the holes. Full details of the construction of the air-driven ultracentrifuges are given by Svedberg and Pedersen (103) and in the publications of Beams and coworkers (114–117). Beams and coworkers (118, 119) have also developed an electrically driven, magnetically supporting vacuum type ultracentrifuge.

Methods of Measurement. Methods based on the refraction principle have assumed great importance for measurements of the sedimentation processes taking place in the ultracentrifuge because many substances do not exhibit light absorption suitable for photographic measurement of concentration gradients. The refraction methods have chiefly been developed in their theoretical aspects by Lamm (see (103)). The two applications of the refraction principle are the scale method and the slit method (Toepler 'Schlieren' method).

In the scale method, (103, page 254) a uniform transparent scale is photographed through the centrifuge cell. The concentration gradient in the cell gives rise to an optical inhomogeneity which causes a deformation of the projected scale. A scale line displacement in the resulting photograph is proportional to the derivative of the refractive index of the solution in the cell at a depth corresponding to the displaced scale line position. The refractive index gradient is proportional to the concentration gradient in the cell since the refractive index, in general, is an approximately linear function of the concentration.

The slit method is the well-known Toepler 'Schlieren' method, which also depends upon the deviation of a beam of light in a concentration gradient. According to Tiselius and coworkers (120), a lens placed as closely as possible behind the centrifuge cell projects an image of a horizontal slit through the cell onto the camera objective. In front of this is a vertically movable screen, having a horizontal edge with which any part of a deviated image of the slit may be cut off. When the screen is properly arranged, any gradients occurring in the cell will give rise to corresponding black bands visible to the eye in the image of the cell. The bands can be recorded photographically.

Various refinements have been devised for the estimation of the molecular weight from the raw ultracentrifugal data. In the diffusion equilibrium method, the molecular weight is more easily evaluated than by the method used heretofore (Chap. VIII, pp. 382-384) from a plot of the logarithm of the concentration (c) against the distance from the center of rotation (x). If the protein solution is monodisperse a straight line is obtained in which the slope is equal to

$$\frac{Me~\omega^2(1-\bar{v}\rho)}{4.606RT}$$

where Me is the molecular weight, ω is the angular velocity, \bar{v} is the partial specific volume, ρ is the density of solution, R is the gas law constant, and T is the absolute temperature. Departure from a straight line relationship is evidence of a polidisperse protein.

The difficulties in calculating the molecular weight from the sedimentation constant (S) obtained by the sedimentation velocity method is due to the non-spherical nature or the possible hydration of proteins in solution. The means of estimating the corrections that have to be applied in order to take into account the effect of the non-spherical nature or the hydration on the frictional resistance to sedimentation are described by Svedberg and Pedersen (103) and by Oncley (121). Hydration, in particular, is emphasized by Oncley.

4. Molecular Weights from Diffusion and Viscosity. New measurements of the diffusion coefficients of certain proteins, using the refractometric scale method to measure the concentration gradients, have been carried out by Lamm and Polson (122) and by Neurath and coworkers (123–126). The coefficient of diffusion is a function of the size and also of the shape of the molecule. Only in the case of spherical molecules are the coefficients, according to theory, inversely proportioned to the radius. All proteins have smaller diffusion coefficients than those calculated from their molecular volume on the assumption that they are spheres. The dissymmetry number of Svedberg (see Chap. VIII, page 392), which is also the ratio of the theoretical to the actual diffusion coefficient, is ordinarily interpreted in terms of the departure from spherical shape, although interpretation on the basis of hydration has been attempted (121).

Diffusion and viscosity are closely related physical phenomena.

Molecular weights may be calculated from a mathematical relation involving diffusion and sedimentation velocity and by functions combining diffusion and viscosity. Because of this, Neurath and coworkers (125, 126) have made considerable use of viscosity measurements as an aid to the interpretation of the shape of protein molecules. In this work they have employed the equations derived by Simha (127) for viscosity, from hydrodynamic considerations, taking into account the influence of Brownian motion. These are:

For rods: $\eta_s = \frac{f^2}{15(\log 2f - 3/2)} + \frac{f^2}{5(\log 2f - 1/2)} + \frac{14}{15}$

For disks: $\eta_s = \left[\frac{16}{15} \, \frac{(1/f)}{\tan^{-1}(1/f)} \right] G$

Where η_s is the specific viscosity (Chap. IX, page 452), G, the fraction of the volume occupied by the solution, and $f = a_1/a_2$. In the above, a_1 represents the long axis and a_2 the short axis of the particle assuming that it has the shape of an ellipsoid of revolution.

A summary of the molecular weight data derived from diffusion and viscosity measurements is given in Table II, page 1110.

5. Molecular Size and Shape from Dielectric Constant Dispersion Curves. Molecules which possess a permanent electric moment show an orientation polarization as well as distortion polarization. High frequency and high viscosity hinder the orientation of the dipole molecule in an alternating field and consequently the dielectric constant decreases with increasing frequency of the alternating potential. This phenomenon is known as anomalous dispersion. A plot of the dielectric constant of a protein solution against the frequency of the alternating potential yields a dielectric constant dispersion curve which has the 'S' shape of the dissociation curve of a weak acid, when plotted as a function of the pH.

The dielectric dispersion curves are interpreted by a combination of Debye's dipole theory (128) and Perrin's theory of the hydrodynamics of elongated particles (129). According to Debye's theory, the dielectric constant drops from an upper static value, ϵ_0 , to a lower limit, ϵ_{∞} , as the frequency of measurement is increased to such a point that the dipoles can no longer follow the alternating field. The frequency ν_{ϵ} at which the dielectric constant is midway

Table II

Molecular Weight and Molecular Constants of Proteins from Diffusion and Viscosity Measurements

		Diffusion		η_s	Viscosity² Rods³ Disks³			
Proteins I	D ₂₀ ×10 ⁷	7 M_{D}	Source	$\overline{CV_0}$	f/f_0	Mv	f/f_0	Mv
Ovalbumin	7.76	43,800	(122)	5.13	1.21	39,000	1.26	34,400
Carbon monoxi	de							
hemoglobin	6.90	63,000	(122)					
Serum albumin	6.45	67,100	(122)	5.07	1.20	73,200	1.25	64,500
				to	to	to	to	to
				6.91	1.30	65,400	1.38	54,500
Gliadin	6.72	27,500	(122)					
Lactoglobulin								
pH = 5.0	7.18	40,000	(122)	5.60	1.24	43,200	1.29	38,200
pH = 9.5	6.78	39,600						
Erythrocruorin	1.96	1,634,000	(122)					
Pseudoglobulin	1 4.60	167,000	(125)	8.27	1.36	203,400	1.47	161,700
				to	to	to	to	to
				9.63	1.41	174,100	1.55	132,500
Pepsin A ¹	1							
(most soluble	9.17		(125)	6.00	1.26	39,900	1.32	26,800
	}	35,500						
Pepsin B ¹								
(least soluble	9.96		(125)	5.23	1.22	26,800	1.27	23,800

 $D_{20} = \text{Diffusion coefficient at } 20^{\circ}.$

 M_D = molecular weight from diffusion and sedimentation velocity.

 η_s/CV_0 = limiting slope of the specific viscosity-volume concentration curves.

 $f/f_0 = \text{dissymmetry constant}$.

Mv = molecular weight from viscosity and diffusion.

¹ Diffusion coefficient values of pepsin and pseudoglobulin are corrected to 25°.

² All viscosity data taken from Neurath, Cooper, and Ericksson (125).

³ Calculated from equations of Simha (127).

between the upper and lower values of ϵ (inflection point of the dispersion curve) is given by the relation

$$\nu_c = \frac{1}{2\pi\tau} \; \frac{(\epsilon_{\infty} + 2)}{(\epsilon_0 + 2)}$$

where τ is the relaxation time.

For a spherical particle, the relaxation time is also given by the relation

$$\tau = \frac{3\eta M \imath}{RT}$$

where η is the viscosity, M is the molecular weight, v is the partial specific volume, R is the gas law constant, and T is the absolute temperature. The equations given above for τ serve to indicate how the dielectric constant dispersion may be used to calculate molecular weights. For elongated particles, Perrin's equations (129) are employed. Estimation of the molecular weights of proteins from the dielectric constant dispersion have yielded the following values: zein, 38,000 (130); hemoglobin, 67,000 (131); gliadin, 38,000 (132); lactoglobulin, 40,000 (133a). The dispersion curves of other proteins such as the serum proteins have not found a ready interpretation (133b).

- 6. Molecular Size and Shape from Double Refraction of Flow. The orientation of asymmetrical particles in the stream lines of a flowing liquid is a function of the shape and size of the particle. Development of the theory and the application of double refraction of flow to the estimation of the size and shape of proteins has been reviewed by Mehl (134). Because of the incompleteness of the theoretical development, the results obtained by this method have only qualitative significance.
- 7. Molecular Size from x-ray Spectrograms. Estimation of the size and shape of protein molecules from x-ray data has been considered by Crowfoot (135) and by Fankuchen (136).

The two techniques by which x-ray diffraction patterns are obtained are by irradiation of a powder or of a single crystal with a monochromatic x-ray beam. The data obtained by the powder method with proteins have only been susceptible of interpretation in cases of simple cubic or hexagonal close-packed systems as is the case with tobacco seed globulin and tomato bushy stunt virus (137). The diagrams, obtained by the single crystal method, are more readily susceptible to interpretation in terms of the molecular size.

The values of the unit cell dimensions are obtained from measurement of locations of the x-ray reflections. From the volume of the unit cell and the density of the protein, the mass of the unit cell is computed. Dividing this by the mass of the hydrogen atom $(1.66\times10^{-24}$ gm.) gives the molecular weight of the components of the unit cell. This may be the molecular weight (if the unit cell consists of a single molecule of the protein) or a multiple of the

² Ross, W. F., J. Biol. Chem., 127, 179 (1939) finds that the minimum molecular weights for the product from pancreatic digestion of CO hemoglobin are 1060, based on the iron content, and 1300, on the non-dialyzable residue.

molecular weight of the protein. In evaluating the number of molecules in the cell, recourse must be had to non-x-ray data such as the symmetry of the space groups or from the ultracentrifuge.

Only in the simplest crystals, are the x-ray data alone sufficient to determine the molecular size and the structure. The three crystalline proteins, insulin, horse methemoglobin, and lactoglobulin have been extensively studied by the single crystal crystallographic method. In the case of insulin, Crowfoot points out that the comparison of Patterson projections for wet and air-dried insulin provide strong evidence in favor of the Svedberg molecule in the crystalline protein. In the case of the wet lactoglobulin crystals, the molecular weight of the unit cell indicated the presence of eight Svedberg units plus a large proportion of water of crystallization. A summary of the calculations of the molecular weights from x-ray data made by Fankuchen are given in Chapter IX, Table II. The values found from the x-ray analysis, in general, are in fair agreement with those obtained by other methods.

8. Colloidal Osmotic Pressure of Serum Proteins. The colloidal osmotic pressure of proteins (chiefly albumin and globulin) of blood serum may be expressed, as a first approximation, as the sum of a function of the albumin concentration and a function of the globulin concentration:

$$C.O.P. = f'A + f''G$$
 (6)

or

$$C.O.P. = k'A + k''G \tag{7}$$

where k' and k'' are empirical conversion constants for multiplication. C.O.P. is expressed in mm. of water and the concentrations of A and G are in gm. per 100 cc. Numerical values for these constants at 0° have been determined by Keys (138) to be k' = 45.2 and k'' = 18.8 or k'/k'' = 2.4. This ratio, in agreement with that expected from the molecular weights, indicates that serum albumin has about 2.4 times the osmotic activity per gm. exhibited by serum globulin. The C.O.P. of the serum proteins increases more than in simple linear proportion with increasing protein concentration. Equation (7) may be altered to cover more than one level of protein concentration by writing it in the form

Gross C.O.P. =
$$f_c(45.2A + 18.8G)$$
 (8)

At 0° some of the values for f_c corresponding to total protein concentrations (gm. per 100 cc.), P, are: P=1.0, $f_c=0.88$; P=2.0, $f_c=0.92$; P=3.0, $f_c=0.98$; P=4.0, $f_c=1.03$; P=5.0, $f_c=1.09$; P=6.0, $f_c=1.17$; P=7.0, $f_c=1.28$; P=8.0, $f_c=1.45$.

Variations in pH over the range pH 6.9-7.6 will affect the above values by less than 6 per cent. For a limited range, allowance for the effect of temperature on the colloidal osmotic pressure of blood serum may be made by writing equation (8) in the form

C.O.P. =
$$f_e(45.2A + 18.8G) \times \frac{T^{\circ} \text{ (absolute)}}{273}$$
 (9)

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CHAPTER IX

CERTAIN CHEMICAL AND PHYSICAL CHARAC-TERISTICS OF THE PROTEINS

SECTION I. DENATURATION OF PROTEINS

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1. New Denaturing Agents. Two new and useful groups of denaturing agents have been discovered, viz., guanidine salts (86) and detergents such as the long chain alkyl sulfates (87). Certain guanidine salts denature proteins much more rapidly than urea and are effective in lower concentration than urea. The effectiveness of a guanidine salt depends on its anion as well as on its cation. Thus guanidine iodide is more effective than guanidine chloride which in turn is more effective than guanidine carbonate (88).

The exact amount of detergent needed to denature a protein and to keep the denatured protein in solution depends on the nature and concentration of the protein and on the structure of the detergent (87). Most detergents are extremely effective and can denature proteins in dilute solution even when there is present only a fraction of a mg. of detergent per cc. Tobacco mosaic virus is much more resistant to detergents than proteins such as egg albumin and hemoglobin (87). Cytochrome c can be separated from other enzymes by the fact that it is not irreversibly destroyed by sodium dodecyl sulfate (89).

2. Surface Denaturation. The properties of denatured protein in surface films are discussed in several reviews (90, 91, 92, 93, 94) and in Section II of this chapter.

Denaturation at a surface is very rapid. If the concentration of the protein is not too low, the rate of denaturation depends only on the rate of formation of new surface and is largely independent of the protein concentration and the temperature (95, 96, 97, 98). The rate at which new surface is created can be established accurately by the simple and ingenious technique of dipping a roller into a protein solution and rotating it at a known rate (98). Surface denaturation takes place even at a pH away from the isoelectric point at which the denatured protein is insoluble (97, 99, 100).

- 3. Denaturation by Pressure. There has been considerable study of the denaturation by high pressure of various pure proteins, including enzymes and tobacco mosaic virus, and also of the inactivation of many unpurified proteins with biological activity (101, 102, 103, 104). In the case of a complex molecule like tobacco mosaic virus, it is important to know that denaturation procedures, such as application of heat (105) or pressure (104), can cause inactivation before they cause denaturation, that is, the formation of insoluble protein.
- 4. Denaturation by Radiation. When proteins are exposed to ultra-violet light and x-rays, they are denatured and other changes are also brought about, in particular splitting of the protein (106, 107, 108, 109).
- 5. Dissociation by Denaturing Agents. Denaturing agents such as urea, guanidine hydrochloride, and detergents can dissociate hemocyanin (110), hemoglobin (111), tobacco mosaic virus (112, 113), and myosin (114) into smaller units. This dissociation, in some cases, may be brought about by a lower concentration of denaturing agent than is necessary to bring about changes characteristic of denaturation (111, 114, 125).

Conjugated proteins such as hemoglobin (87) and cytochrome c (89) are not only denatured by detergents but, under some conditions, detergents can break some of the bonds between the prosthetic group and the protein. Detergents can dissolve chloroplasts with accompanying denaturation of the protein present (86, 115, 116). Digitonin and bile salts separate the protein from the pigment (117). Sodium dodecyl sulfate splits off the magnesium from the pigment (116) and splits the protein complex into smaller units (117), but leaves the pigment a part of a pigment-protein complex (117).

- 6. Digestibility. Denatured hemoglobin is readily digested by all the proteinases that have been tried and it is therefore a convenient and reproducible substrate for the estimation of proteinases (118). Native hemoglobin, in contrast, is not digested by trypsin (119). The plant proteinases, asclepain (120) and papain (121), digest native hemoglobin at about 1 per cent of the rate at which these plant proteinases digest denatured hemoglobin.
- 7. Shape of Denatured Protein. The increase in viscosity observed when a protein is denatured in urea solution (112) is probably due to the change in shape of the protein molecule brought about when the protein molecule is opened in denaturation (123).

Calculations from new diffusion and viscosity data indicate that the molecule of denatured protein in urea solution is extremely elongated, being some twenty times as long as it is wide (124, 125). The elongated shape of the denatured molecule has also been calculated from viscosity data alone (126). Thus denatured protein is extended in solution as well as at a surface.

Protein fibers have been made by suitable precipitation of denatured protein (127, 128). It has been pointed out that the elongated shape of the individual molecules of denatured protein makes denatured protein particularly suitable for fiber formation and that detergents are desirable reagents to denature and dissolve the protein to be converted into fibers (129).

8. Denaturation and the Properties of Protein Groups-SH Groups. The methods for estimating the SH groups of proteins have been made more sound and convenient. The SH groups of denatured egg albumin are now estimated in the presence of urea, guanidine hydrochloride (86), or detergents such as long chain alkyl sulfates (130). These reagents not only denature egg albumin but keep the denatured protein in solution. One can measure how much porphyrindin (86, 131, 132), ferricyanide, or tetrathionate (133) is needed to oxidize the SH groups, how much p-chloromercuribenzoate is needed to combine with the SH groups (133), how much ferricyanide (130, 133, 134), iodosobenzoate (135), or uric acid reagent (136) is reduced by the SH groups. The SH groups of egg albumin, furthermore, can be estimated without denaturing the protein by measuring how much iodine is reduced by native egg albumin in cold 1 m potassium iodide solution (136).

A great deal of varied evidence has been provided that, in the new procedures, all the SH groups and only SH groups are estimated. When several different procedures were applied to a single sample of egg albumin, the same value for the SH content of the protein was obtained in all cases (133). Egg albumin, however, as usually prepared, is not a single pure protein whose amino acid content is always the same. Differences in the SH contents of egg albumin reported from different laboratories, therefore, may be due to differences in the samples of egg albumin used rather than to differences in the analytical methods employed.

Aggregation and precipitation of denatured egg albumin interfere with the reactions of the SH groups of the protein with ferricyanide (134, 136, 137) and iodoacetate (138). The earlier estimations of the SH content of egg albumin were carried out with

suspensions of the coagulated protein rather than with solutions of the denatured protein. The value of the SH content of egg albumin obtained by these earlier procedures was about half the correct value.

Ferricyanide and iodoacetamide abolish all the SH groups of neutral denatured egg albumin even in the absence of reagents such as urea and alkyl sulfates if care is taken to avoid precipitation (130, 136). Ferricyanide also oxidizes all the SH groups of egg albumin denatured by heat or shaking if the ferricyanide is present during the denaturation, so that it can oxidize the SH groups before aggregation and precipitation take place (134, 139). Porphyrindin, a stronger oxidizing agent than ferricyanide, can oxidize all the SH groups even of precipitated denatured egg albumin if the porphyrindin is present in sufficiently high concentration (140).

The exact reactions of the SH groups of denatured egg albumin depend on which denaturing agent is present. Denatured egg albumin gives a strong pink color with nitroprusside in guanidine hydrochloride solution, a weaker color in urea solution, and practically no color in detergent solution (133). The uric acid reagent, a weaker oxidizing agent than ferricyanide, oxidizes the SH groups of neutral denatured egg albumin in urea solution but not in detergent solution or in the absence of denaturing agents (136).

The SH groups of free cysteine and of the cysteine peptides formed by slight peptic digestion of egg albumin are more easily oxidized than the SH groups of denatured but unhydrolyzed egg albumin (136).

Although native egg albumin does not reduce ferricyanide or give a nitroprusside test, the SH groups of egg albumin can be abolished by treating the native protein with dilute iodine (141), dilute permanganate (136), or concentrated hydrogen peroxide (136). The concentration of hydrogen peroxide needed to abolish the SH groups is thousands of times greater if the peroxide is added to native egg albumin than it is if the peroxide is added to denatured egg albumin. Some of the SH groups of egg albumin can be abolished by treating the native protein with iodoacetamide (141).

Native egg albumin, the SH groups (or their precursors) of which have been oxidized by a stoichiometric amount of iodine in cold potassium iodide solution, has the same sedimentation rate as untreated egg albumin (136). In other words, there is no combination of two SH groups from two different albumin molecules to form an

S-S group. The SH groups of native egg albumin probably exist as neighboring pairs.

Tobacco mosaic virus is an SH protein of the egg albumin type. It gives a pink color with nitroprusside and reduces porphyrindin only when denatured (113). But its SH groups can be abolished by treating the native virus with iodine (142). Native tobacco mosaic virus, the SH groups of which have been oxidized with iodine, stimulates tobacco plants to produce virus with the normal SH content (142).

Iodoacetate abolishes the SH groups and the enzymic activity of papain, although papain gives a pink color with nitroprusside only when the protein is denatured (143, 144).

Urease, like myosin and lens protein, has some SH groups that are reactive in the native protein and more SH groups that become reactive only when the protein is denatured. There are two different types of reactive SH groups in native urease. One type gives a pink color with nitroprusside. When this SH group is combined with p-chloromercuribenzoate, the urease is still active. The second type of SH group of native urease does not give a nitroprusside test. When this second type of SH group is combined with p-chloromercuribenzoate, the urease is inactive (145).

The SH groups of native myosin that give a nitroprusside test and reduce porphyrindin do not give these SH tests in the presence of ammonium salts and guanidine hydrochloride. If guanidine hydrochloride is added and the myosin is denatured, the SH tests are obtained even in the presence of ammonium salts and glycine (146).

The change into an insoluble form that myosin undergoes on being frozen is similar, as shown by suitable SH and solubility tests, to the change that myosin in muscle undergoes during contraction. Various drugs that can cause contraction of denervated muscle also increase the rate at which isolated myosin is changed into an insoluble form by freezing (147).

9. Other Protein Groups. Other protein groups have not as yet been studied with the same detail as the SH groups. What evidence there is, however, indicates that the reactions of other protein groups, like the reactions of SH groups, depend on what protein and what reagent is used, on whether the protein is native or denatured, on whether the denatured protein is aggregated or not, on what denaturing agent is present, and on whether the protein is partially hydrolyzed or not.

There are tyrosine groups in pepsinogen that are oxidized by the phenol reagent when the protein is denatured but not when the protein is native (148). Urea promotes the oxidation of tyrosine and tryptophane groups of denatured protein just as it promotes the oxidation of the SH groups of denatured protein (136). In this connection it is interesting that urea promotes the oxidation even of free tyrosine and tryptophane (136).

It is important for the theory of protein structure to know by what structural mechanism denaturation makes protein SH, S-S, tyrosine, and tryptophane groups more reactive, and why the SH groups of native egg albumin react with iodine and not with ferricyanide. Unfortunately, the present experimental information does not, as yet, permit any certain conclusions. The structural mechanism will become more clear as more varied reactions of many different protein groups become known. It has been suggested that the SH groups of egg albumin are inaccessible to ferricyanide in the interior of the native molecule and become accessible when the molecule becomes denatured and opened (134, 139). It has been suggested also that the sulfur groups that exist in denatured egg albumin as SH groups, exist in the native protein in some non-SH form (86, 149, 150). Finally, it is possible that the SH groups of native egg albumin are free and accessible but less reactive than the SH groups of denatured egg albumin just as the SH groups of denatured egg albumin are less reactive than the SH groups of cysteine or of slightly hydrolyzed egg albumin (141).

10. All or None Character of Denaturation. When egg albumin is gradually denatured by urea or by shaking in the presence of ferricyanide, the fraction that is denatured, as shown by the solubility test, no longer has any SH groups, whereas the fraction which is not denatured has had no SH groups oxidized by ferricyanide (134, 139). In general, if denaturation in solution is followed by two different methods and there is no complication of dissociation or aggregation, when the protein is half denatured by one test it is half denatured by the other test. So far as one can tell by the tests used, an individual molecule is usually either completely native or completely denatured and any molecules in intermediate stages are rare.

Measurements with the ultracentrifuge indicate that native thyroglobulin, in a solution not containing salt, is in equilibrium with an extended but not denatured form called alpha protein (151, 152). It is claimed that this alpha form is an intermediate

stage in the dissociation and denaturation of thyroglobulin and in its digestion by papain. More experiments are required to bring the conclusions from the experiments with salt-free thyroglobulin in harmony with the general knowledge about digestion and denaturation in solution.

- 11. Denaturation of Proteins in the Solid State. There has been very little study of the reactions in general and of the denaturation in particular of proteins in the solid state, despite the fact that a significant fraction of all cellular proteins is not in solution. It has been confirmed that when protein crystals are heated they still appear crystalline in form (153). X-ray examination of heated crystals would show in a rough way to what extent the structural changes characteristic of denaturation have taken place despite the superficial crystal form of the heated protein, and to what extent, if any, the bonding of the atoms in the original crystal has modified or limited the changes characteristic of denaturation. Temperature has roughly the same effect on the rate of denaturation of protein in precipitated form as it has on the rate of denaturation in solution (154). This suggests that the unit that undergoes denaturation in the precipitate is similar to the unit that undergoes denaturation in solution.
- 12. Reversal of Denaturation. The denaturation of chymotrypsin (155), of pepsinogen (148) and, under certain conditions, of thyroglobulin (151) and surface denatured pepsin (91, 156) are readily reversible.

In general, native protein prepared from denatured protein has the same sort of properties as untreated native protein, although in some cases quantitative differences have been found (157, 158, 159, 160, 161, 162, 163).

The curve relating pH to the equilibrium between native and denatured pepsinogen indicates that denaturation of pepsinogen involves combination of the protein with two additional hydroxyl groups (148).

Bacterial luminescence can be inhibited reversibly by raising the temperature. The temperature at which luminescence is quenched reversibly to a given extent is lowered by lipid-soluble narcotics and raised by pressure. These results have been interpreted to mean that there is in living bacteria a reversible equilibrium between native, active luciferase and denatured, inactive luciferase which is shifted to the denatured side by heat and narcotics and to the native side by pressure (164). It is likely that many cases of reversible thermal inhibition of biological processes due to reversible denaturation in living cells will be demonstrable if irreversible destruction is avoided by not using temperatures higher than the temperature necessary for thermal inhibition and by cooling the material just as soon as the thermal inhibition is established.

13. Heat of Denaturation. The heat of denaturation of pepsinogen calculated from the effect of temperature on the equilibrium between native and denatured pepsinogen at pH 7.0 is 31,000 calories (148). The heat of denaturation of luciferase, calculated from the effect of temperature on the reversible heat quenching of bacterial luminescence, is 55,000 calories (164). It should be remembered that the heat of denaturation calculated from the effect of temperature on the denaturation equilibrium depends on the pH and the denaturing agent present and includes the heats of all the changes that take place in connection with the denaturation procedure.

Heats of denaturation by alkali have been measured by direct calorimetry and found to be 100,000 calories for hemoglobin (165) and 85,000 calories for pepsin (166). In view of some uncertainties in the corrections that had to be made, it cannot as yet be said how accurate the final results are. It is clear, however, that there will soon be greatly increased information about the heat of denaturation under various conditions.

Experiments on the denaturation of protein and its reversal, on the properties of the reversed protein, and on the properties of denatured protein at a surface should preferably be carried out with a protein such as chymotrypsinogen which is pure and stable and whose denaturation is completely reversible. This is especially true if thermodynamic information is desired. Unfortunately, most

¹ The control of luminescence can be represented schematically as follows:

$$A_{n} = 17,000 \text{ cal.} \quad A_{n}^{*} \rightarrow A_{n} + h\nu$$

$$u = 55,000 \text{ cal.} \quad A_{d}$$
(II)

wherein native luciferase, A_n , becomes excited to A_n^* in an oxidative reaction, and then emits light in a reaction independent of temperature. The amount of active, native luciferase depends on the equilibrium (II) between the native and denatured forms. Thus at any temperature, the actual light intensity is the net result of the two simultaneous reactions, (I) and (II).

experiments have not been carried out with such desirable material. It is interesting that native hemoglobin formed by the conversion of some of the hemoglobin denatured by trichloroacetic acid was denatured at a different rate from normal, untreated, native hemoglobin. In contrast, native hemoglobin, prepared by converting all the hemoglobin denatured by salicylate back into native hemoglobin, was not distinguishable from untreated hemoglobin (159).

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SECTION II. SURFACE TENSION AND FILMS

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SPREADING OF PROTEINS

- 1. Technique. Proteins may be spread in monolayers in different ways. The technique used by Gorter and Grendel has already been described (page 428). Modifications are given by Neurath and by Bull (26). Devaux's (27) technique consists in dissolving the protein in water thus forming a surface monolayer spontaneously in less than a second. The process of spreading at the air-water interface proceeds with sufficient energy to build up a pressure of 8 dynes per cm. in 1 minute, 10 dynes in 5 minutes, and 15 dynes in 15 minutes. This method may be used to study the concentration of protein as a function of the rate of formation of the monolayer. Langmuir and Schaefer (28) use a nickel sheet about 0.010 inch thick, as long as the width of the tray, and as wide as its depth. The protein solution is spread on this sheet from a micropipette and the sheet is lowered at a uniform rate through the cleaned water surface. The pressure that is built up during formation of such a monolayer retards the rate of formation somewhat so that some of the solution is dispersed into the substrate. When the protein solution is introduced as a uniform strip across the tray, the spreading proceeds very efficiently on the surface of the upflow of water that follows the lowering of the sheet. Hughes and Rideal (29) produce monolayers by placing a weighed amount of dried protein on a water surface. This method is limited to such proteins as spread readily when dry. The maximum spreading usually occurs at or near the isoelectric point.
- 2. Thickness of Protein Monolayers. The thickness of protein monolayers is usually given as 10 Å units. Various techniques have been employed to determine this. In the case of certain stearates, Blodgett and Langmuir (30) deposited successive monolayers on a solid surface and measured the optical thickness. For barium stearate the thickness per layer was found to be 24.4 Å units. The thickness of protein monolayers may be determined similarly by depositing the protein on a barium stearate step-plate. Between pH 2-7 the thickness of a pepsin monolayer is given as 16 Å units. Another technique consists in forming a large number of

¹ In this connection see Astbury, W. T., and Bell, F. O., Cold Spring Harbor Symp. Quant. Biol., 6, 117 (1938).

protein monolayers by dipping a plate through monolayers on water (31). These strips are birefringent. The protein chains lie roughly parallel to the direction of the movement of the plate.

The evidence indicates that the thickness of protein film is approximately the same for all proteins. When values considerably greater than 10 Å units have been obtained, it is probable that more than a single monolayer was present. The value assigned to the side chain spacing of the β -keratin configuration is about 10 Å units. If all side chains were oriented on the same side of the surface, the film thickness should be only about 5 Å units. Since the polar side chains are directed towards the water and the apolar chains towards the air, it is not expected that the thickness of the monolayer would have this low value. The indications are that most proteins contain about an equal number of polar and apolar groups. On this basis the minimum area per amino acid will be about 31.5 square Å units. If all groups were oriented on the same side of the plane, the area should be about 15.7 square Å units. The 10 Å value is in line with the above data.

- 3. Force-Area Curves. By use of a surface balance, data may be obtained for plotting force (dynes/cm.⁻¹)—area (M²/mg.) curves for proteins. Curves for pepsin and egg albumin resemble one another closely but differ from those of insulin and gliadin. The curves appear to depend on the particular protein under investigation. When the pressures are low (0.2–2 dynes), the slope of the pressure-area curve increases rapidly. This is probably due to packing of the protein molecules and change in the orientation of the side chains of the amino acid residues. The change in orientation is probably complete when, with further pressure, the force-area curve becomes linear.
- 4. Reversed Compressibility. Protein monolayer films show a high reversible compressibility. A monolayer of insulin may momentarily be decreased 80 per cent in area and it will reexpand to its original area. Recovery is not complete if the film is exposed to a high pressure (35+dynes per cm.) for several minutes. Repeated compression, even with low pressures, will shift the lower portion of the pressure-area curve of a protein such as gliadin. It is possible that repeated compression leads to elimination of some water from the protein film and the "dehydrated" film responds less to compression. Another possibility is that under continued pressure there may be interaction between some of the free groups in the side chains.

5. Viscosity of Protein Monolayers. Schaefer (32) classifies protein monolayers into 3 classes: Star-like (egg albumin, pepsin, to-bacco seed globulin), rough circular (trypsin, papain, wheat gliadin), and smooth circular (insulin, casein, zein). The patterns are obtained by forming a monolayer, followed by the expansion of its central region with a spreading oil, which reveals the degree of cohesion by which the units of monolayer are joined together. The viscosity range of the rough circular pattern is from 0.009 to 0.081 C.G.S. units at F=2 dynes. The smooth circular types have a viscosity range of 0.001 to 0.004 C.G.S. units at F=2 dynes while the viscosity range of the star-like form ranges from 0.024 to 120 C.G.S. units at F=2 dynes. The viscosity of the monolayers varies with the value of F.

The quantitative determination of the viscosity of monolayers is made by means of a platinum disk suspended by a fine wire to a torsion head. The disc lies in the surface of the water. By turning the torsion head the disc can be made to oscillate and the damping can be used to measure the viscosity (33). The viscosity of smooth and circular patterns may be measured by the oscillation method. Since the star-like patterns usually have a high viscosity, the aperiodic method is preferably used. The same viscosimeter is employed. A comparison is made of the relative moment of the suspended disc in contact with the monolayer with the initial displacement of the torsion head as a function of elapsed time.

- 6. Anisotropy. When the protein monolayer is compressed for several minutes by the motion of a barrier which raises the pressure to about 35 dynes per cm. so that some collapse occurs, a permanent anisotropic condition is produced. When the protein monolayer is compressed equally in all directions as by use of piston oil, it shrinks uniformly toward its geometrical center and is then isotropic.
- 7. Influence of pH and Other Factors on Spreading. The area of protein films is greatly influenced by pH. Maximum areas per unit weight occur at the isoelectric point and in very acid or alkaline substrates with a marked minimum in between. This is true for all "globular" proteins. Proteins with rod-like molecules, as myosin and fibrinogen do not spread unless some of the CONH bonds are first broken. Heat causes a marked decrease in the spreading tendency. This may be restored by the action of proteinase. The spreading of proteins into unimolecular films is rapid and complete if the salt content is about 0.1 N. If the effective protein

charge is high, spreading is very slow. Anions decrease the time if the protein charge is positive, cations if it is negative. Multivalent ions have a stronger effect than monovalent ions. The above are general statements. The magnitude of the effect of pH and concentration of inorganic salts will depend on the number and strength of the ionic groups of the protein molecule.

8. Surface Potential Measurements. For this purpose a movable radioactive electrode is placed above the surface, a reversible electrode is placed in the solution contained in the trough, and a potentiometer-electrometer circuit is used thus permitting measurements of the surface potential difference between the air-liquid interface to be made. Descriptions of the apparatus are given by Harkins (34) and by Philippi (35). The surface potential difference between a clean surface and a film covered surface is given by the equation

$\Delta V = 4\pi\mu n$

where ΔV = potential difference in millivolts, n = the number of molecules per unit surface, and μ = the vertical component of the dipole moment of the film molecules. This equation neglects the forces between film and water molecules, the rearrangement of dipoles and ions of the underlying solution, and the interaction between neighboring film molecules. If no rearrangement takes place, the value for μ should remain constant within a certain compression range. Variations are probably due a change of the angle of tilt of the film molecules to the surface. On the basis of the above equation, the surface potential, when plotted against film areas, should be a straight line provided no rearrangement of the film molecules occurs. This is usually the case when the pressures are small. With larger pressures, the side chains may be forced out of the plane of the surface and hence a change of dipole moment occurs.

9. Nature of Monolayers. The upper surface of the film is made of nonpolar groups which face the air. The lower layer which faces the water contains the polar groups. The central layer contains the peptide groups. The polar groups are probably attached to the water molecules by means of hydrogen bridges. The marked insolubility of protein monolayers, even under high compression, results from the great length of the polypeptide chains in the monolayers. The apparent large compressibility of protein films appears to result from a squeezing out of certain weakly hydro-

phobic amino acid residues from positions at the air-water interface into an underfilm where they contribute little to the surface pressure, although they are still attached through the polypeptide chains to the more hydrophobic residues that remain in the overfilm. A correlation can be made of the compressibility curves of proteins to their composition. The amino acids with side chains having a hydrophobicity less than that of the C2H5-group determine the compressibility in the range from F=1 to F=3 while those more hydrophobic determine the areas at F=25 (36). The tendency of a protein to form monolayers decreases with the number of Svedberg units contained in the molecule. It would be interesting to study the spreading of proteins on solutions such as urea in order to determine the influence of these compounds on the dissociation of proteins into units of lower molecular weight (37). The denaturation of protein films is discussed in Section I of this chapter.

A number of reviews (38) have appeared since the original section on protein films was written by Gorter. These cover the subject more adequately than the present space permits.

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SECTION III. ELASTICITY OF PROTEINS

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1. Elasticity of Fibrous Proteins. The elasticity of fibrous proteins is a property of protein plus water or other small polar molecules. When the stretched protein is absolutely dry, it shows practically no tendency to contract. According to Astbury (21), the molecular structure of fibrous proteins is that of a system of long polypeptide chains running parallel to the fiber axis. The polypeptide chains are linked through their side chains to form polypeptide "grids" of the type shown diagrammatically in Fig. 1.

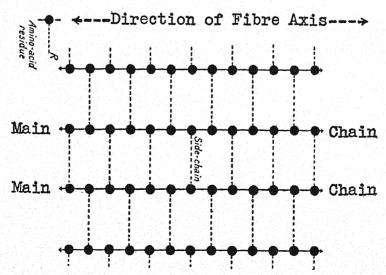


Fig. 1. Diagrammatic representation of a keratin "grid" in the β -configuration. The unstretched or α -configuration may be obtained from this by throwing the paper into a series of folds whose crests run parallel to the side-chains.

(Astbury, W. T., and Bell, F. O., Brit. J. Dermat, and Syph., 52, 173 (1940).)

In the unstretched state, the grids are not flat but are buckled. They have a wave-like contour because the main-chains in equilibrium are constricted into a series of folds lying in planes transverse to the side-chains. Where the protein is stretched or otherwise forcibly deformed, the main-chains are pulled out straight and the grids become flat. This is a reversible, elastic, intra-molecular transformation that roughly doubles the length. When the stretching force is removed, the polypeptide chains refold, and the protein recovers its normal length. The phenomenon depends on an interchange between the short normal form of α -keratin and the same molecule pulled out straight as in β -keratin. Water is necessary before the main-chains can be pulled out straight against the contractile forces of the side-chains. The above principles are involved in the creasing of trousers and the waving of hair. When the fiber is bent, the outer arc is stretched more than the inner. If this is done while the protein is wet and heat applied, the shape of the protein will be maintained for considerable time. If the protein fiber is stretched in cold water to about 50 per cent extension and then treated with steam or treated with alkali in the cold, it acquires an increased range of elastic recovery and will contract to a length about one-third shorter than its original unstretched length. It is probable that the steaming or other treatment affects the side-chains in such a way that the main-chains are more free to contract.

According to Woods (22, 23), the elastic behaviour of protein fibers such as hair is due to the occurrence of "elastic phases" that extend inside of the fiber cells as well as between them. The first phase, termed K1, is that part of the fiber substance which under ordinary stretching in cold water is responsible for extensions up to about 20 per cent. It is not well organized crystallographically. The second phase, termed K2, is "crystalline" and is involved in extensions greater than about 20 per cent. It is at about this point that α -keratin progressively changes to β -keratin. However, interactions between the two phases may take place and this complicates the problem of accounting for the elastic phenomena. Young's modulus should be a function of the properties of phase K₁. Under certain conditions, however, phase K₂ may also be involved. Young's modulus is, therefore, not entirely a function of the total water-content of the fiber as might be expected. Up to about 6 per cent, water has comparatively little influence on Young's modulus; after that a proportionality exists. Between 20°

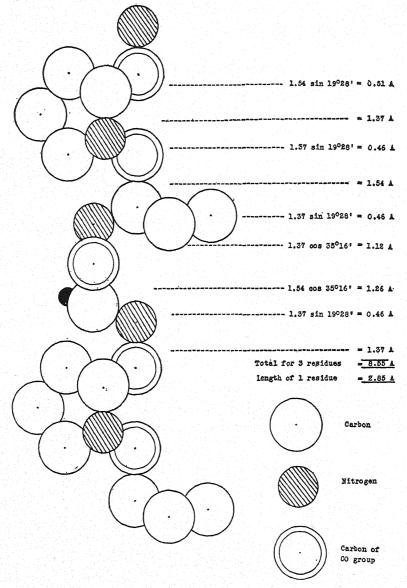


Fig. 2. Schematic representation of the intramolecular pattern along the collagen chains.

(Astbury, W. T., First Procter Memorial Lecture, Int. Soc. Leather Trades' Chemists, London, 1939.)

and 100°, Young's modulus of dry fibers changes linearly with temperature. This relation is not so well defined when the fibers are wet. It is not yet possible to relate fiber structure to Young's modulus except in a general way.

2. Elasticity of Myosin. Astbury (24) and Astbury and Dickinson (25) point out a similarity between myosin and the labile or supercontracting form of keratin. The elastic range of oriented myosin, on stretching, is similar to that of keratin. Myosin contracts (supercontracts) spontaneously in hot water or cold dilute alkalies in the same way as keratin does after breakdown of certain cross-

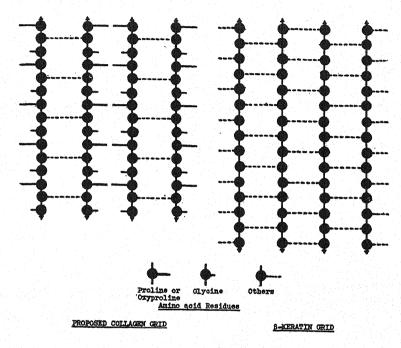


Fig. 3. Schematic comparison of the collagen grid with the β-keratin grid. (Astbury, W. T., First Procter Memorial Lecture, Int. Soc. Leather Trades' Chemists, London, 1939.)

linkages of the polypeptide grid. When moist myosin is pressed between plates at ordinary temperature, the side-chains are oriented perpendicular to the plane of flattening in the same way as keratin does after breakdown of the cross-linkages. Schematic representation of the intramolecular pattern along the collagen chains is shown in Fig. 2. A schematic comparison of the collagen and β -keratin grids is given in Fig. 3. In this grid the important side-chains occur on the same side of the backbone at intervals of three, instead of intervals of two as is characteristic of the β -keratin grid. The average side-chain spacing is somewhat greater than in β -keratin while the backbone spacing is smaller.

It is pointed out that the chief component of the x-ray photo-

graph of muscle arises from myosin oriented along the length of the fibrils. In resting muscle the myosin grids are already folded in the α -configuration and contraction takes place by virtue of further folding. The contraction of the myosin grid is actuated by means of a cycle of chemical events that changes the state of combination of the cross-linkages of the grid.

While the views of Astbury and coworkers may be accepted as a schematic and tentative explanation of the relation between the elastic properties and the chemical structure of proteins, they should not be taken too literally and are by no means final. A final explanation will only be at hand when the complete molecular

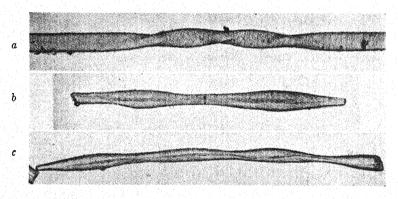


Fig. 4. a. Photomicrograph of pili torti. b. Photomicrograph of monilethrix. c. Photomicrograph of monilethrix showing incipient twists.

(Hellier, F. F., Astbury, W. T., and Bell, F. O., Brit. J. Dermat. and Syph., 52,

173 (1940).)

configuration of elastic proteins in their various states becomes known.

3. Pili Torti and Monilethrix. Pili torti or twisted hair is a rare congenital abnormality (6). Clinically it resembles monilethrix, a condition of the hair in which the nodes can be seen strung regularly or irregularly along the shaft of the hair, the internodular portions being relatively colorless, atrophied, and thinned. In pili torti the hairs are not beaded, but are flattened and, at irregular intervals, completely twisted through 180° around their long axis. Photomicrographs of hairs characteristic of both conditions are shown in Fig. 4. Neither pili torti nor monilethrix involves any molecular disturbance recognizable by x-ray diffraction that is different from the keratin type of structure. The two conditions are histological irregularities. These are, however, only spasmodic

variations of the normal. In curly or crinkly hairs, the hair-shaft is commonly twisted about its axis, first one way and then the other. In merino wool, the hair-shafts twist through approximately 180° and back for every complete wave-length of the crimp. The causal mechanism is apparently associated with a curvature in the follicle and partly with rhythmic rotatory movements of the latter. The load-extension curve of pili torti is much the same as that of keratin fibers and very like that of merino wool. The abnormal hairs are about 10 per cent weaker than normal hairs when dry and about 25 per cent weaker when wet. The coarser, more ribbon-like fibers are quite brittle. It is apparent that in pili torti we are not dealing with a difference in the general grid pattern characteristic of keratins but rather some spasmodic variation of the normal structure of hair.

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SECTION V. VISCOSITY OF PROTEIN SOLUTIONS

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The renewed interest in the viscosity of protein solutions is due to the derivation of new theoretical relations between this variable and the size and shape of large molecules. The new interest has led to the recent publication of a considerable amount of data on the change of viscosity with concentration of a large number of well defined undenatured proteins (24–29) and of urea and heat denatured proteins (30, 31). The viscosity of proteins differs so greatly that it may serve to characterize individual proteins. Fahey and Green (24) found this to be the case for the various serum proteins. Except in very dilute solution, the curves of relative or specific

viscosity plotted against concentration are not linear. Treffers (32) has shown that plots of the relative fluidity (reciprocal of the relative viscosity) against protein concentration, yield straight lines even up to concentrations of 150 gms. of protein per liter. The linear

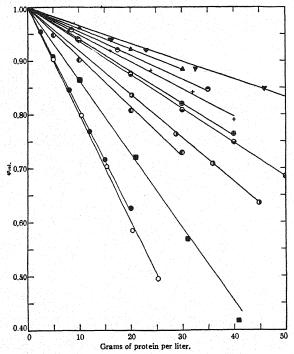


Fig. 1. Fluidity concentration curves of representative protein solutions. ▼, oxyhemoglobin; ▲, CO-hemoglobin; ⑤, homarus hemocyanin; +, trypsin; ⊗, octopus hemocyanin; ⑥, thyroglobulin; ⑥, horse pseudoglobulin; ■, PII, ○, PIII (horse globulin fractions); ⑥, gliadin; ⑥, beef globulin.

Treffers, H. P., J. Amer. Chem. Soc., 62, 1405 (1940.)

relation of relative fluidity (ϕ_r) to protein concentration (C) may be represented by the simple mathematical equation,

$$\phi_r = 1 - KC$$

where K is a constant characteristic of the protein. Treffers found that the linear relationship also holds for mixtures of proteins when there is no specific interaction among the proteins. He suggests that this principle be applied to the analysis of such protein mixtures as the albumin and globulin of the blood serum.

The viscosities of a number of representative proteins plotted as the relative fluidity against concentration are shown in Fig. 1.

There exists no completely satisfactory theoretical derivation of a viscosity equation, other than the Einstein equation (Chap X, page 455) for the case of rigid, spherical, electrically neutral particles, whose size is large compared to the size of the solvent molecules. Various attempts have been made to derive viscosity equations for large non-spherical molecules which have the form either of an

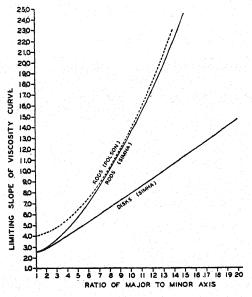


Fig. 2. Relation between viscosity and the axial ratio of protein molecules according to Simha and Polson. The upper two curves refer to the viscosity equations of Polson and Simha for molecules having the shape of prolate ellipsoids (rods); the lower curve to Simha's equation when solved for the shape of oblate ellipsoids (disks).

(Neurath, H., Cooper, G. R., and Erickson, J. O., J. Biol. Chem., 138, 411 (1941)

ellipsoid (33–37) or of a rod (38, 39). The most recent and what appears to be the best developments are the equations of Simha (37) for the case of ellipsoids and of Huggins (39) for rod-like molecules, which relate the viscosity to the ratio of the long to the short axis of the molecule. The limiting forms of the equations derived by Simha for short rods and for disks are given in Chapter VIII, page 1109, where they have been considered in the discussion on

¹ A theory of viscosity that applies with great accuracy to normal liquids has been derived by Kincaid, J. F., Eyring, H., and Stearns, A. E., *Chem. Rev.*, 28, 301 (1941) from the treatment of the mechanism of absolute reaction rates by means of statistical mechanics. In developing this theory, diffusion in liquids was treated as a rate process.

molecular weights of proteins. The relation between viscosity and the axial ratio of protein molecules is shown in Fig. 2.

Huggins (39) has considered the case of rigid and of randomly kinked long-chain molecules like the aliphatic paraffins. For rigid rod-like molecules, he developed an equation which in low concentrations simplifies to

$$\eta_s = \frac{\pi}{24,000} N l^2 a n^2 C$$

where η_s is the specific viscosity, N is Avogadro's number, l is the distance between centers of the submolecules, i.e., the repeating units of the rod-like molecule, a is the radius of the submolecule, n is the number of repeating units, e.g., CH_2 groups in a paraffin, and C is the concentration in submoles per liter, i.e., the primary molar concentration of Staudinger (See Chap. IX, p. 460).

For chain molecules which are kinked at each submolecule, with a uniform bond angle but otherwise in a random way, Huggins obtained the limiting equation,

$$\eta_s = \frac{\pi}{3000\sqrt{5}} N l^2 a B_{\infty} n C.$$

 B_{∞} is a symbatic function of the bond angle which has the value two for tetrahedral bond angles. The other terms in the equation have the significance given above. The equation for randomly kinked molecules has the same form as the Staudinger equation

$$\eta_s = knC$$

which was derived empirically from an examination of the viscosity data of synthetic polysterenes of varying chain length.

Previous to the work of the last named authors, Polson (25) had made use of an empirical modification of the Kuhn equation (38), based on relating the molecular weights derived from sedimentation and diffusion data to the viscosity of the proteins, to estimate the axial ratios of proteins.

While the new derivations of Simha and of Huggins, represent a distinct advance in the theory, they do not contain a complete solution of the viscosity problem of large molecules for all conditions. The equations they have derived are chiefly of value as relating to the limiting values of the viscosity in dilute solution. From the results of recent trials, the Simha equations appear to be the most adequate to represent the viscosity-concentration

curves of most proteins because of their low asymmetry. The values of the molecular weights and asymmetry constants obtained for a number of proteins by Neurath and coworkers (27), on application of the Simha equations, have already been discussed in the section (Chapter VIII, page 1110) on the molecular weights of proteins.

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SECTION VII. HYDRATION (BOUND WATER) OF PROTEINS IN SOLUTION

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Some progress has been made along a number of lines toward the solution of the problem of the nature of the hydration of proteins. A distinction appears to exist between the properties of water in protein systems containing less water than about 50 per cent of the weight of the protein (termed here low water content-protein systems), and protein systems with greater amounts of water (high water content-protein systems). In the low water content systems, the water appears to be in a condition similar to that found in crystal hydrates, where the water may fill in spaces not

capable of being occupied by the stable components of the crystal lattice.

As in the case in crystal hydrates, the partial molal heat contents and the partial molal entropies of the water have negative values in low water content-protein systems (26). The decrease in entropy indicates a less random distribution of the water than is characteristic of water in ordinary solutions. It may be inferred that a small amount of water is intimately associated with the protein (bound) and possesses special thermodynamic properties.

Further evidence for the binding of water by protein (gelatin) in low water content-protein systems has been furnished by examination of the infra red spectra (27–29). Ellis and Bath (27, 28) state that the infra red absorption spectrum of a protein is relatively simple, in spite of the complexity of the protein molecule, and can be readily interpreted in terms of the vibrations of CH, NH and C=0 groups. Oven dried gelatin in the region between 1 and 2.5 μ contains the following assignable bands.

There is a band at $1.50~\mu$ which is due to the first overtone of the valence vibration of the NH and NH₂ groups and a band at $1.72~\mu$ which is due to the first overtone of the CH, CH₂, and CH₃ groups. Bands due to the combination of fundamental valence vibrations and deformation vibrations occur as follows: NH, at $2.05~\mu$; NH₂, at $2.18~\mu$; and C—H at $2.28~\mu$. When the gelatin is placed in a water saturated atmosphere, it absorbs about 35 per cent of its weight of water and the absorption spectrum is changed as follows. The $1.5~\mu$ band is greatly diminished in intensity and the $2.05~\text{and}~2.18~\mu$ bands are weakened. The bands at $1.72~\text{and}~2.28~\mu$ retain about the same intensity but are slightly shifted to higher regions. Absorption of D₂O causes almost complete suppression of the $1.5~\mu$ band.

The interpretation by Ellis and Bath of the spectral changes produced by the water is that the dipole moment oscillation of the NH-groups, constituting a part of the inter-molecular hydrogen bridge, CO—NH, is reduced because this bridge is broken by the water molecules which themselves become bridged to the NH-groups. In producing this change, D₂O is far more effective than H₂O. Changes also take place in the infra red absorption spectrum of the water present in the gelatin which show that there is a decrease in the freedom of the water molecules and indicate that most of the molecules are bound, the binding force being provided by hydrogen bridges.

Sponsler, Bath and Ellis (30) have endeavored to estimate the

number of water molecules coordinated with gelatin from an evaluation of the hydrogen bond forming groups in the amino acid residues which can be calculated from the analytical composition of gelatin. Assuming a chain molecule of 288 amino acid residues with a molecular weight of 27,000, they arrived at the value of 750 water molecules per molecule of gelatin. It may be noted in passing that the above contains various unproved assumptions.

Fricke and coworkers (31, 32) have found that the addition of water to gelatin results in increased dielectric polarizability and strong anomalous dispersion (see Chapter VIII, page 1109) which reach their maximum values at approximately 55 per cent water. The addition of the first 10 to 20 per cent water has little effect on the dielectric properties of the gelatin and thus it is believed to be very strongly bound. The large effect on the dielectric properties of the gelatin is interpreted as being produced by a polymolecular layer of loosely bound water.

A volume contraction occurs in gelatin-water systems until the water content reaches the value of about 0.6-0.7 gm. per gm. of gelatin (33, 34). Heymann (33) has suggested that this water may be regarded as bound because it has a higher density than free water. In such systems, the volume contraction cannot solely be ascribed to the water and consequently, it does not provide a quantitative measure of hydration. All of the procedures that have been cited above only provide qualitative evidence of hydration.

The density of protein crystals suspended in salt solutions is less than the density of the dry protein. Adair and Adair (35) suggest that the low density is due to water of hydration and, if the water in the crystal is combined with the protein by covalent bonds, the hydration should be carried over with the protein when the latter goes into solution. In further support of their hypothesis, they state: "It has been shown that a number of inorganic ions, including phosphate and ammonium, diffuse freely into the protein crystal, and the environment of a protein molecule in the crystal thus corresponds closely to the environment when in solution. It is therefore reasonable to suppose that the degrees of hydration in the crystalline form and in solution are at least approximately equal."

Quite another interpretation of the data of Adair and Adair seems quite plausible. The estimated degree of hydration of the protein crystals varies greatly in different electrolyte media. For horse hemoglobin it varies between 0.166 to 0.344 gm. H₂O per gm. of

protein, for edestin between 0.063 and 0.143, and for serum albumin between 0.182 and 0.344. The variation in the hydration and the high permeability of the protein crystals would seem to put the systems composed of protein crystals and their mother liquor in the same category as that of an elastic gel immersed in an aqueous solution, where the water uptake is generally assumed to be an osmotic effect and not due to primary valence forces. If this be true, when the protein dissolves, the crystals cease to exist as a separate phase and the osmotic force disappears. (see Chapter XIV, page 802, and Chapter IX, page 472.) Consequently, there need be no relation between the amount of water in the crystals and the hydration of proteins in solution.

In high water content-protein systems hydration in the sense of non-solvent (bound) water is probably of minor significance in determining the characteristics of protein solutions. The thermodynamic properties of protein solutions, particularly those containing electrolytes, often differ greatly from those predicted for ideal solutions. "Bound" water is commonly defined as the amount of water required to compensate for the deviation of the activity of water from the measurement expected in an ideal solution (26). The interionic attraction theory of electrolytes (36) and the extension of this theory to dipolar ions (Chapter XVI, page 919) offers an alternative explanation of the variation in thermodynamic properties in terms of the action of electrical forces.

Using a sensitive vapor pressure method and the method of freezing point lowering, Chandler (26) has examined the deviations from the behavior in simple solutions produced by various types of crystalloids in protein-containing solutions. A measurement greater than the empirically determined value of the pure substance alone he terms a positive deviation and a measurement less than the empirical value a negative deviation. A typical illustration of his data is given in Table I for the effect on the freezing point depression.

The data of Table I show that the changes taking place in solution are the results of the interaction of all components and not the effect of the colloid alone. Changes of the same kind may be produced in purely crystalloidal solutions. The direction of the deviations are predicted by the interionic attraction and dipolar ion theories.

Chandler found an increase in the heat content of gelatin containing solutions. This is opposed to the concept of molecular

Table I

Deviation in Freezing Point Depressions as Various Components

are Added to Solutions

System	Solute	Molal Freezing Point Depression		Devia-
		Calc.	Obs.	tion
	moles	°C	°C	Per cent
 (a) H₂O—5 per cent gelatin (b) H₂O—5 per cent gelatin—0.8 M 	0.80 glucose	1.910	2.00	4.7
glucose	0.40 KCl	3.315	3.28	-1.1
 (c) H₂O-5 per cent hemoglobin (d) H₂O-5 per cent hemoglobin- 	0.69 glucose	1.905	2.07	8.6
0.4 M glucose	0.22 KCl	3.375	3.24	-4.0
(e) Wheat sap ("hardened")	0.44 glucose	1.895	2.18	15.1
(f) Wheat sap ("hardened")	0.31 KCl	3.360	3.08	-8.3
(g) H ₂ O-0.4 M KCl-0.8 M	5 per cent gel-			
glucose	atin	1.860	$(-0.037)^*$	_
(h) H ₂ O-0.5 M alanine-0.5 M	5 per cent gel-			
glucose	atin	1.860	(-0.015)*	_
(i) H ₂ O-0.5 M glycine-0.74 M	5 per cent gel-			
glucose	atin	1.860	(-0.013)*	-

* Observed elevations of freezing point. These rose slightly when gelatin was added.

(Chandler, R. C., Plant Physiol., 16, 273 (1941).)

orientation of water which should decrease the heat content. The concept of non-solvent (bound) water has no physical meaning when the measured deviation is negative as is the case in particular when the electrolyte is KCl. Chandler concludes that the part played by proteins in altering the thermodynamic properties of water appears to be due chiefly to their electrical properties and their effect may be of minor importance compared with that of the crystalloidal components of the solution.

Weismann (37) and Blanchard (38) have reviewed the published evidence for the hydration of proteins, and both conclude that no sound evidence exists for the occurrence of non-solvent (bound) water in protein solutions of high water content.

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SECTION VIII. PROPERTIES OF CRYSTALS

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1. Crystallization. As has been pointed out previously, many of the salts of amino acids are very useful for purposes of crystallographic identification. Kirk and coworkers (50) have prepared photomicrographs of the picrolonic acid salts of certain of the amino acids. These are shown in Fig. 1.

CHARACTERIZATION OF PICROLONATES OF AMINO ACIDS

Alanine: The picrolonate crystallized along the edge of the drop in bundles of long yellow acicular prisms with pointed tips, shown in Fig. 1(1). Anisotropic, parallel extinction, negative elongation. $N_1 = 1.575$, $N_2 = 1.580$.

Arginine: Three types of crystal were obtained as follows:

- a) Fine straight needles, almost colorless to yellowish-brown, formed along the edge of the drop, shown in Fig. 1(2). Anisotropic, parallel extinction, positive elongation. $N_1 = 1.716$, $N_2 = 1.580$.
- b) Yellow-brown fern-like isotropic crystals forming on the ends of the straight needles, yielding rather large groups. N = 1.578.
- c) Occasional rosettes of light-yellow needles formed slowly from surrounding oil droplets. Anisotropic, parallel extinction, positive elongation.

Aspartic acid: Two types of picrolonates were obtained.

- a) Coarse yellow acicular prisms formed along the edge of the drop. These were somewhat soluble in water. Accurate refractive index measurements could not be made. Anisotropic, parallel extinction, positive elongation $N_1 < 1,527, 1,527 > N_2 > 1.512$.
- b) Rosettes and tufts of fine acicular prisms, yellowish-brown in color, formed in the center of the drop. Anisotropic, parallel extinction, negative elongation. N_1 and N_2 greater than 1.740.

Cysteine: Chains of brush-shaped bundles of short acicular prisms, yellowish-brown in color, formed around the edge of the drop, or tightly packed bundles and rosettes in the center of the drop. Anisotropic, parallel extinction, negative elongation. N_1 and N_2 greater than 1.740. On standing, the center rosettes, instead of growing in diameter, fill out to give the appearance of dark circular disks.

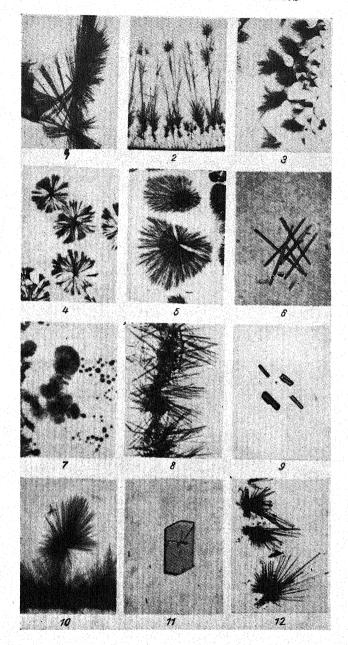


Fig. 1. Picrolonates of amino acids. 1. Alanine; 2. Arginine; 3. Cystine; 4. Dibromotyrosine; 5. Diiodotyrosine; 6. Glutamic acid; 7. Histidine; 8. Hydroxyvaline; 9. Isoserine; 10. Lysine; 11. Phenylalanine; 12. Valine.

(Dunn, R., Inouye, K., and Kirk, P. L., Mikrochemie, 27, 154 (1939).)

Of the greatest importance is the fact that many of the virus proteins have been obtained in crystalline form both in vitro and in vivo (51). A photomicrograph of a preparation of bushy stunt

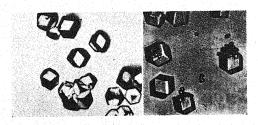


Fig. 2. Left-hand: crystalline material possessing a high specific activity and obtained from bushy stunt virus. Preparation 3. Right-hand: crystalline material possessing a low specific activity and obtained from bushy stunt virus. Preparation 6a. ×89.6. (Photographs by J. A. Carlile.)

(Stanley, W. M., J. Biol. Chem., 135, 437 (1940).)

virus that possessed a high specific activity and one that was partially inactivated by chemical and heat treatment are shown in Fig. 2. The electron microscope has permitted further study of the

Cystine: Characteristically shaped tufts of fine brownish-yellow needles, shown in Fig. 1(3) formed along the edge of the drop. Anisotropic, parallel extinction, positive elongation. $N_1 = 1.600$, $N_2 = 1.548$.

Dibromotyrosine: Two types of picrolonates were obtained.

a) From dilute solution, sheaves and bundles of fine yellowish-brown needles grew along the edge of the drop. In the center were formed spreading sheaf-shaped rosettes of similar acicular prisms. With increased concentration these grew into circular rosettes shown in Fig. 1 (4). Anisotropic, parallel extinction, positive elongation. $N_1 > 1.740$, $1.616 > N_2 > 1.549$.

b) Irregular groups of nearly colorless acciular prisms with obliquely truncated ends were formed. Anisotropic, oblique extinction at approximately 44°, negative elongation. $N_1 < 1.549$, $N_2 > 1.580$.

Dichlorotyrosine: The picrolonate formed a clear oil which, after heating and partial cooling, yielded a fine granular precipitate. On further cooling to room temperature, this precipitate dissolved and dense spherical rosettes of nearly fibrous crystals formed. Anisotropic, parallel extinction, positive elongation. $1,740 > N_1 > 1.698, 1.633 > N_2 > 1.618$.

Diiodotyrosine: A clear oily liquid formed first as with dichlorotyrosine. On partial cooling a dark granular precipitate formed which was converted into roughly circular rosettes of fine yellowish-brown needles, shown in Fig. 1(5). Anisotropic, parallel extinction, positive elongation. N₁ and N₂ could not be measured.

Glutamic acid: The picrolonate formed small elongated prisms shown in Fig. 1(6). Anisotropic, parallel extinction, negative elongation. $N_1 = 1.574$, $N_2 = 1.596$.

Glycine: The picrolonate formed dense rosettes of fine acciular prisms, yellowish-brown in color. Anisotropic, parallel extinction, positive elongation. $N_1=1.616$, $N_2=1.531$.

Histidine: Precipitation of histidine is almost immediate and probably quantitative, with formation of yellowish-brown, circular or fan-shaped rosettes of fine

size and shape of viruses (52). An electron micrograph of tobacco mosaic virus is shown in Fig. 3. It is likely that it will not be possible to crystallize all of the viruses especially those having a complex chemical makeup, at least for the present.

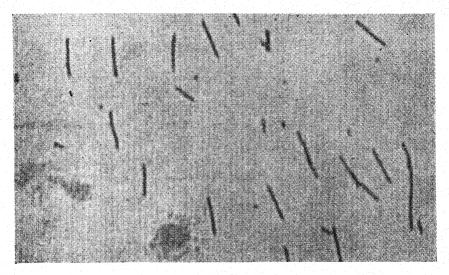


Fig. 3. Electron micrograph of tobacco mosaic virus. (Stanley, W. M., and Anderson, T. F., J. Biol. Chem., 139, 325 (1941).)

From a study of x-ray powder diffraction photographs of crystalline tobacco mosaic virus, Wyckoff and Corey (53) conclude that the patterns are exactly those expected from true crystals composed of large molecules. According to Bernal (54) the solid needleshaped particles seen under the microscope are really in a liquid

acicular prisms shown in Fig. 1(7). As little as 0.001 mg. of pierolonic acid in solution gave a precipitate with a minute amount of histidine. Anisotropic, parallel extinction, positive elongation. $N_1=1.616$, $N_2=1.557$.

Hydroxyproline: On standing several minutes, fan-shaped rosettes of fine acicular prisms with pointed ends formed along the edge of the drop. Anisotropic, parallel extinction, positive elongation. $N_1 = 1.658$, $N_2 = 1.493$.

Hydroxyvaline: Oily drops were formed which, on agitation, produced chains of irregular rosettes of coarse acicular prisms, yellow-brown in color, shown in Fig. 1(8). Anisotropic, parallel extinction, positive elongation. $N_1 = 1.56$ (approximately), $N_2 = 1.54$ (approximately). Immersion media dissolved the crystals, making accurate measurement impossible.

Isoleucine: The picrolonate formed chains of rosettes of almost fibrous yellow prisms. Anisotropic, parallel extinction, positive elongation. $N_1 = 1.610$, $N_2 = 1.520$.

Isoserine: Three types of crystal were obtained.

a) The most characteristic crystals were short, yellow, highly refractive six-sided

crystalline state and should be regarded as liquid or paracrystals. The form of the virus crystals in living cells may be altered from that of hexagons to needles by addition of acids (55). The reverse may also occur (56). It is possible that the various crystalline forms of the viruses form an unbroken series of crystals. The hexagons have been considered as being true crystals (57) since they appear to possess 3-dimensional regularity. The needles apparently possess only 2-dimensional regularity and hence may not conform to the precise definition of a crystal. The crystallinity of proteins is not a sufficient criterion of purity or chemical homogeneity. However, when the crystalline state is taken together with other criteria of homogeneity and homomolecularity and all of the criteria point in the same direction, in the present state of our knowledge the protein must be considered as homomolecular.

Moody, Proescher, and Carr (58) report that, when dried blood is heated above 700° F., the charred residue assumes a specific structure. Whether or not specific structures can be obtained for pure proteins when treated similarly is not yet evident. It would be interesting to investigate this lead further.

According to Langmuir (59), the crystallization of proteins is to be regarded as an example of unipolar coacervation (micelles having like charges) which must involve attractive forces. Coulomb attrac-

prisms with almost square ends. They formed slowly on standing. These are shown in Fig. 1(9). Anisotropic, parallel extinction, sign of elongation not determinable. $N_1 > 1.740$, N_2 , approximately 1.70.

b) Short, almost fibrous brown needles appeared along the edge on cooling. Anisotropic, parallel extinction, positive elongation. $N_1 = 1.608$, $N_2 = 1.520$.

c) Sheaves of short, brown acicular prisms. Anisotropic, parallel extinction, positive elongation. $N_1 = 1.660$, $N_2 = 1.529$.

Leucine: Oily liquid formed which, on agitation, yielded unsymmetrical rosettes and bundles of long, yellow acicular prisms. Anisotropic, parallel extinction, positive elongation. $N_1 = 1.617$, $N_2 = 1.527$.

Lysine: The picrolonate formed clusters of small brownish-yellow needles which on further growth, yielded rather long reddish-brown crystals arranged in irregular rosettes, as in Fig. 1(10). Anisotropic, parallel extinction, positive elongation. $N_1 = 1.645$, N = 1.520.

Methonine Gradual solution of the yellow oil which formed at first, slowly yielded large sheaves of coarse, dark, pointed, acicular prisms similar to tyrosine picrolonate. Anisotropic, parallel extinction, positive elongation. $N_1 =$ approximately 1.62, $N_2 = 1.494$.

Norleucine: The preparation yielded crystals very slowly. Standing for 30 minutes produced dense rosettes of rather coarse prismatic needles having a characteristic banded appearance. Anisotropic, parallel extinction, negative elongation. $1.740 > N_1 > 1.658$, $1.740 > N_2 > 1.658$. Crystals dissolved in the immersion media.

Norvaline: (a) The oily liquid first formed crystallized slowly in bundles of

tion rather than long range van der Waals forces appear to be involved. The writer feels that the phenomenon of crystallization is a universal one and the same factors that operate to determine crystal structure of small molecules are also concerned in large molecules such as the proteins. The difference may be one of degree rather than one of kind.

2. Crystal Structure. (a). Amino Acids. X-ray diffraction studies of glycine, the diketopiperazine of glycine, and alanine have recently been carried out. According to Albrecht and Corey (60) glycine crystals are built upon a simple monoclinic cell $(a_0 = 5.10 \text{ Å}, b_0 = 11.96 \text{ Å}, c_0 = 5.45 \text{ Å}, and } \beta = 111^{\circ}38')$ containing four molecules. All atoms are in general position of the space group $C_{2h}^5 - P2_{1/n}$. The following are the atomic parameters: oxygen (I), x = 0.805, y = 0.410, z = 0.740; oxygen (II), x = 0.360, y = 0.360, z = 0.610; carbon, x = 0.575, y = 0.380, z = 0.560; methylene carbon,

yellowish-brown needles, the rate increasing noticeably on slight agitation. Anisotropic, parallel extinction, positive elongation.

(b) In addition there were formed rosettes of coarse acciular prisms. Anisotropic, parallel extinction, negative elongation. $N_1 = 1.684$, $N_2 > 1.74$.

Phenylalanine: Two types of crystals were obtained.

(a) The usual method of preparation yielded banded rosettes of brown needles. Anisotropic, parallel extinction. Crystals were not suitable for determination of sign of elongation and refractive indices.

(b) On standing, dilute solutions yielded yellow, elongated, six sided plates, shown in Fig. 1(11). Anisotropic, extinction position along shortest diagonal. N_1 and $N_2 > 1.74$.

Proline: From concentrated solution, yellowish-brown dendrites of fine needles formed slowly on standing. Anisotropic, parallel extinction. $N_1 = 1.530$, $N_2 = 1.605$.

Serine: The picrolonate exhibited two crystal forms:

(a) Yellow-brown needles formed immediately from the oily drop and grew into long slender acicular prisms. Anisotropic, oblique extinction at 35-40°, positive elongation. $N_1 = 1.567$, $N_2 = 1.530$.

(b) Varying the concentration sometimes yielded rosettes of light-yellow needles. Anisotropic, parallel extinction, negative elongation. Refractive indices were not measured because of rapid solution in the immersion media.

Tryptophane: On cooling the solution, the picrolonate formed as a highly refractive isotropic mass of indefinite form. N=1.712.

Tyrosine: The picrolonate formed as imperfect rosettes of pointed acicular prisms, dark in color and having a twisted appearance. Anisotropic, oblique extinction at $35-37^{\circ}$, positive elongation. $N_1 = 1.596$, $N_2 = 1.529$.

Valine: The picrolonate crystallized in imperfect rosettes of long acicular prisms yellowish-brown in color, shown in Fig. 1(12). Anisotropic, parallel extinction, negative elongation. $N_1 = 1.549$, $N_2 > 1.740$.

dl-a-Amino-n-valeric acid: The picrolonate formed along the edge of the drop as a chain of intermingled sheaves of rectangularly tipped acicular prisms. Anisotropic, parallel extinction, negative elongation. $N_1=1.685$, $N_2>1.740$.

¹ This is based on a density value of 1.607 for glycine.

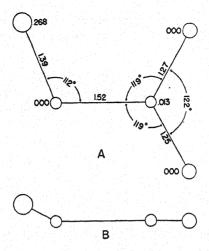


Fig. 4. The glycine molecule viewed (A) perpendicular to a plane containing the two oxygen and the α-carbon atoms, and (B) parallel to this plane.
(Albrecht, G., and Corey, R. B., J. Amer. Chem. Soc., 61, 1087 (1939).)

Fig. 5. A view of the glycine structure parallel to the a-axis of the crystal, showing single and double layers of molecules held together by hydrogen bonds. The 3.05 Å bonds connect atoms of the molecules A and B, whereas the 2.93 Å bonds connect the N and O₁ atoms of A with the O₁ and N atoms of the molecule behind B. The 2.88 Å bonds are likewise tapered to indicate that they connect with atoms in molecules in front of or behind those shown.

(Albrecht, G., and Corey, R. B., J. Amer. Chem. Soc., 61, 1087 (1939).)

x=0.565, y=0.365, z=2.80; nitrogen, x=0.800, y=0.410, z=0.245. The structure consists of nearly flat glycine molecules held together by hydrogen bonds between adjacent nitrogen and oxygen atoms to form continuous layers throughout the crystal. The atomic arrangement indicates a zwitterion structure of the glycine molecule in the crystal. Models showing the structure of glycine are given in Figs. 4 and 5.

The crystals of 2,5-diketopiperazine (61) are built upon a simple monoclinic unit having $a_0 = 5.19$ Å, $b_0 = 11.50$ Å, $c_0 = 3.96$ Å, and $\beta = 83^{\circ}$ and containing two molecules. All atoms are the general positions of the space group $C_{5_2h} - P2_{1/a}$ with the following param-

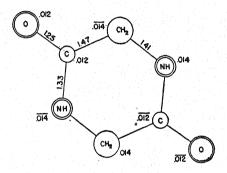


Fig. 6. The diketopiperazine molecule as viewed perpendicular to a median plane. Dimensions are in Ångström units.

(Corey, R. B., J. Amer. Chem. Soc., 60, 1598 (1938).)

eters: oxygen, x=0.160, y=0.368, z=0.885; carbon, x=0.320, y=0.430, z=0.705; methylene carbon, x=0.545, y=0.380, z=0.495; nitrogen, x=0.280, y=0.544, z=0.695. The molecule is a nearly flat hexagonal ring possessing a center of symmetry, the angles between all bonds being $120\pm3^{\circ}$. The molecules are held together by hydrogen bonds to form long, parallel chains throughout the crystal. Models showing the structures of diketopiperazine are shown in Figs. 6 and 7.

The crystals of dl-alanine are built upon an orthorhombic unit having $a_0 = 12.04$ Å, $b_0 = 6.04$ Å, and $c_0 = 5.81$ Å, and containing four molecules of the amino acid. The space group is $C_{2v}^9 - Pna$ with all atoms in general positions, giving the following parameters: oxygen I, x = 0.090, y = 0.481, z = 0.134; oxygen II, x = 0.186, y = 0.201, z = 0.000; carboxyl carbon, x = 0.145, y = 0.312, z = 0.161; nitrogen, x = 0.139, y = 0.393, z = 0.576; α -carbon, x = 0.161, y = 0.227, z = 0.408; methyl carbon, x = 0.091, y = 0.017, z = 0.444. A model of dl-alanine is shown in Fig. 8.

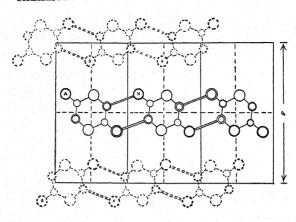


Fig. 7. A view perpendicular to (101) showing chains of molecules held together by hydrogen bonds.

(Corey, R. B., J. Amer. Chem. Soc., 60, 1598 (1938).)

A comparison between the interatomic distances in the three compounds and the normal distances are given in Table I. The shorter than anticipated experimentally determined distances indi-

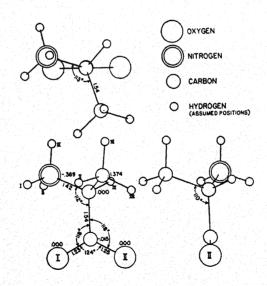


Fig. 8. Three projections of the alanine molecule. Figures given to three decimal places represent the departures in Ångström units of the atoms from the plane of the α -carbon and oxygen atoms; those to two decimal places represent bond lengths in Ångström units.

(Levy, H. A., and Corey, R. B., J. Amer. Chem. Soc., 63, 2095 (1941).)

cated in the table are probably due to the effects of resonance in the respective molecules.

The above crystal structures show the importance of the directional properties of hydrogen bonds between nitrogen and oxygen atoms in determining atomic and molecular arrangements. It appears altogether probable that these directional properties of the hydrogen bond play an important rôle in determining the most stable arrangement of the atoms in protein molecules.

(b). Proteins. X-ray data are now available for insulin (63), pepsin (64), lactoglobulin (65), chymotrypsin (66), hemoglobin (66),

Table I

Comparison Between Some Interatomic Distances, in Ångström Units, Found in Molecules of Glycine, Diketopiperazine, and dl-Alanine and Normal Distances

	$\begin{array}{c} \rm Glycine^1 \\ \pm 0.02 \end{array}$	$\begin{array}{c} {\rm Diketopiperazine^3} \\ {\pm 0.03} \end{array}$	$rac{dl ext{-Alanine}^2}{\pm0.03}$	Normal ⁴
C-0	1.25 and 1.27	1.25	1.25 and 1.23	1.43
C—N C—C	$\substack{1.39\\1.52}$	$\begin{matrix}1.41\\1.47\end{matrix}$	$\begin{array}{c} 1.42 \\ 1.54 \end{array}$	$\begin{array}{c} 1.47 \\ 1.54 \end{array}$
OC—N		1.33		1.47

¹ Hydrogen bond distances between nitrogen and oxygen atoms in the same layer are 2.76 and 2.88 Å; between neighboring layers, 2.93 and 3.05Å. See reference (60).

² The alanine molecules are linked together by a three-dimensional framework of hydrogen bonds, which is responsible for an abnormally close approach (3.64 Å) of methyl radicals of adjacent molecules. The intermolecular interatomic distances between nitrogen and oxygen atoms are 2.88, 2.84, and 2.78 Å. See reference (62).

³ Intermolecular interatomic distance between nitrogen and carbon atom is 2.85 Å. See reference (61).

⁴ Data from Corey, R. B., Chem. Rev., 26, 227 (1940) and Pauling L., The Nature of the Chemical Bond, Ithaca, 1939.

excelsin (67), tobacco seed globulin (68), tobacco mosaic virus (69), and bushy stunt virus (70). Table II, prepared by Fankuchen (71), gives the data for these compounds.

A discussion of the x-ray diffraction methods used in protein studies is given by Warren (72). It is of interest to point out that x-ray studies permit the estimation of molecular weights of proteins. These are in fairly good agreement with those obtained by other methods. The molecular weight calculations are made with the aid of the following equations: If n=the number of molecules per unit cell, M=the molecular weight, N=the Avogadro num-

TABLE II

Protein and References (References are given at the	Insulin	Lactog (6	Lactoglobulin (65)	Chymo- trypsin	Pepsin	Horse Methemo-	Tobacco	Excelsin	Bushy Stunt Virus
end of this section)	(en)	Tabular	Needle	(99)	(04)	(99)	(68)	(20)	(20)
Non X-ray Molecular Weight	35,100 40,900	37, 41,	37,900 41,800	41,000	35,500- 39,200	66,700	300,000	294,000	7,600,000 8,800,000
a in Å (10-8 cm.) c c c c c s s s No. in Å v Vol. in Å s	130 74.8 30.9 90° 30.9 298,000	60 63 110 90° 110 416,000	56 56 130 90° 130 408,000	45 62.5 57.5 112° 53.5 151,000		102 51 47 130° 36 188,000	123 123 123 90° 123 1,850,000	149 86 208.2 90° 208.2 2,670,000	318 318 318 318 90° 318 32,000,000
Nolume per Molecule Density Molecular Weight	50,000 1.315 39,500		51,000 1.30 40,100	75,500 1.31 60,000		94,000 1.270 72,000	460,000 1.287 360,000		16,000,000 1.35 13,000,000
	37,400 R3 7	$P2_12_12_1$	20	$^{54}_{{ m P2}_1}$		66,700 C2 13	322,000 F	305,800 R3	
	a = 44.4 $\alpha = 114^{\circ}28'$ n = 1 Referred to rhombohedral unit cell							a = 85.3 $\alpha = 60^{\circ}36'$ n = 1	
a b b c c sin β c sin β λ s	144 83 34 90° 34 404,000	67.5 67.5 154 90° 154 702,000	67.5 67.5 133.5 90° 133.5 608,000	49.6 67.8 66.5 102° 65 219,000	116 67 461 90° 461 3,580,000	110 63.8 54.2 112° 50.2 352,000			394 394 394 90° 394 61,000,000
Nolume per Moleoule Density Wet Moleoular Weight Space Group Smallest Observed Spacing	67,000 1.28 52,400 R3 2.4	88,000 1.257 67,000 P2,2,2,2	76,000 P4 ₂ 2,	$\begin{array}{c} 2000,000\\ 1.277\\ 84,500\\ P2,2 \end{array}$	54 66,500 1.32 53,000	$176,000$ 1.242 $132,000$ C_2			30,500,000 1,286 24,000,000
	a = 49.4 $\alpha = 114^{\circ}16'$ n = 1								

(Fankuchen, I., Ann. New York Acad. Sci., 41, 157 (1941).) See also Crowfoot, D., Chem. Rev., 28, 215 (1941).

ber, ρ = the density of the crystal and V = the volume of the unit cell then

$$\frac{nM}{N} = V\rho \tag{1}$$

$$n = \frac{NV}{M} \rho \tag{2}$$

$$M = \frac{NV}{n} \rho \tag{3}$$

If the molecular weight, M, is known, the number of molecules per unit cell can be calculated from equation (2). If the number of molecules per cell is known, the molecular weight can be calculated from equation (3). In general, M cannot be obtained from x-ray data alone. Using equation (3), it is possible to calculate a series of possible molecular weights for comparison with determinations made by other methods. Often the lattice and the symmetry of the crystal will impose certain restrictions upon n and consequently upon M. The molecular weight of a protein from x-ray data is one of the series of values calculated with the aid of equation (3) that agrees best with the ultracentrifuge values. This involves a value of n that is compatible with the symmetry of the crystal. The numerical value of M is uncertain to the same extent as the value of the density that is used in the computation.

Certain difficulties may arise in attempting to use the above equations. The density to be used is the density of the crystal in the medium in which it is studied i.e., for dry crystals the density in air, and for wet crystals, the density in the solution. In order to measure densities, immersion in some liquid is necessary. The density may depend on the medium used to immerse the crystals (73). Variations in the cell dimensions exist when the crystals are studied in different solutions (74). The molecular weights of proteins in the anhydrous state, obtained from ultracentrifuge data (75), are compared with the molecular weights determined from x-ray measurements on dry crystals. The density value of the dry crystal (or the wet crystal) must be accurate. However, due to the uncertainties of the water content of the proteins and inadequate information as to hydration of the crystals, the determinations may involve errors. The number n must be an integer, usually a small one. A set of molecular weights is obtained by dividing the cell molecular weight by the possible values of n. The maximum value is the cell molecular weight. In the case of insulin there is only one molecule per unit cell. The value chosen for n is the one that gives a molecular weight value in best agreement with the molecular weight determined by other methods such as the ultracentrifuge, chemical analysis, osmotic pressure, etc. It is possible that some erroneous deductions as to the size and symmetry of the protein unit in the crystal will be made since in some protein crystals the unit may possibly be different from that indicated in the ultracentrifuge or under other conditions for the estimation of molecular weights.

3. X-ray Data and Protein Structure. Extensive use has been made of x-ray data to interpret protein structure. This may be justified from a qualitative but not from a quantitative standpoint. In order that the structure of polypeptides and proteins can be given with certainty, it is necessary that information as to the interatomic distances and bond angles of all of the atoms in the molecule be available. As has already been shown, such data are available for several amino acids only. Any theory of protein structure must agree with x-ray evidence. At the present time the lack of adequate x-ray evidence does not permit the converse statement to be made. Free rotation about single valency bonds would permit peptide molecules to assume a variety of stereochemical configurations. It is to be expected that free rotation is greatly restricted due to steric requirements of the side chains. This consideration will greatly limit the number of possible configurations (76). According to Neurath, the β -keratin is a fully extended uniplanar polypeptide with amino acid side-chain residues in alternate normal directions and hydrogen atoms in a spiral progression about the main chain. Contraction of the main chain as a result of rotations about single bonds produces a folded polypeptide of the α-keratin type. However, due to the spatial requirements of the side chains, free rotations about C-C and C-N bonds must be limited. The folding is probably not strictly co-planar but 3-dimensional with side-chain orientation preserved. Astbury (77) is inclined to consider that the intramolecular folds of the proteins are in essence always of the β -type.

At the suggestion of Professor H. S. Taylor, Becker (78) has modified the Fischer-Hirschfelder atomic models so that they are more adaptable for the study of intramolecular rotation about single bonds. It involves the use of the ordinary dressmaker's snap-fasteners in place of the single-bond pegs. The "female" half

of each fastener is countersunk into the single-bond face of the atom to a distance below the surface of the atom equal to the distance of the "male" half. The "male" parts of the fasteners are soldered together in pairs, back to back, and so serve the same function of a universal joint as the tapered peg of the original model, but more satisfactorily. There is much greater freedom of rotation and, in addition, a remarkable degree of stability is obtained with even the most complex structures. A folded structural model is represented diagrammatically by

This structure affords adequate room for the accommodation of side-chains. These project up and down at right angles to the main chains on alternate sides of the axis of the chain. Adjacent chains may be joined by double hydrogen bonding. The structure is essentially a series of alternating "Y" and "A" amino acid residues with a repeating unit along the chain of 4.5Å. The structure can be further folded in the planes of the backbone (perpendicular to the previous folding) and still maintain the up and down configurational arrangement of the side-chains. While the above scheme may not actually represent the configuration of proteins, it does indicate that possibly there may be numerous configurations that protein molecules, in their various states, may assume. Caution should be exercised in accepting any theory of protein structure until data, amply supported by the use of all techniques that can be applied to the problem, become available.

The term "globular proteins" has been used where reference is made to macro-crystalline proteins. Since many of the proteins do not appear to be spherical but rather rod-shaped, the suggestion has been made (79) that the term "corpuscular proteins" be used. The use of this term might imply that there is a distinction between fibrous and globular proteins. There is reason for believing that fibrous and globular proteins are closely related, if not actually similar, in structure. The structures of proteins range from the fully-extended β -proteins to the effectively spherical forms such as

insulin (80). Artificial fibrous proteins may be produced from denatured globular proteins. The writer believes that nothing is gained by the employment of terms that attempt to draw fine distinctions between the various types of protein molecules. The terms "crystalline" and "non-crystalline or amorphous" serve amply, in the present state of our knowledge, to characterize the physical state of proteins. Crystalline proteins are those that possess physical structures that lend themselves to crystallographic characterization. Others should be termed amorphous until they can be prepared in a crystalline state. If and when protein molecules can be characterized structurally which implies adequate information as to bond angles, resonance, shape, and size, there may be no need of differentiating between the amorphous and the crystalline state.

Crowfoot (81) has recently reviewed the x-ray work on protein crystals.

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SECTION IX. MAGNETIC AND DIAMAGNETIC PROPERTIES

By CARL L. A. SCHMIDT

(Division of Biochemistry, University of California Medical School, Berkeley)

1. Properties of Hemoglobin. A number of important papers dealing with the magnetic properties of the hemoglobins have appeared. These throw further light on their physico-chemical behavior. Corvell, Stitt, and Pauling (5) have made magnetic measurements at 24° of ferrihemoglobin (acid methemoglobin) and certain other hemoglobin compounds. The paramagnetic molal susceptibilities (×10⁻⁶) are: ferrihemoglobin (Hb⁺), 14,040; ferrihemoglobin hydroxide (HbOH) (alkaline methemoglobin), 8,340; ferrihemoglobin fluoride (HbF), 14,610; ferrihemoglobin cyanide (HbCN), 2,610; ferrihemoglobin hydrosulfide (HbSH), 2,140. The values of the paramagnetic part of the molal susceptibility correspond to the following values of the effective magnetic moment per heme, expressed in Bohr magnetons: ferrihemoglobin, 5.80; ferrihemoglobin hydroxide, 4.47; ferrihemoglobin fluoride, 5.92; ferrihemoglobin cyanide, 2.50; ferrihemoglobin hydrosulfide, 2.26. For ferrihemoglobin and its fluoride, these correspond to five unpaired electrons per heme, indicating essentially ionic bonds; for the cyanide and hydrosulfide to one, indicating essentially covalent bonds; and for the hydroxide to three, indicating bonds of an intermediate type.

Taylor and Coryell's (6) values of the paramagnetic susceptibilities per formula weight of the iron in various hemoglobins (×10⁻⁶) are: cow, 12,290; horse, 12,260, sheep, 12,390; human, 11,910. The values are in c.g.s.u. at 25°. The magnetic moments (Bohr magnetons) of these hemoglobins (assuming independent iron atoms) are: cow, 5.435; horse, 5.43; sheep, 5.46; human, 5.35. On the basis of the above data it is now possible to determine hemoglobin concentrations magnetometrically with high precision.

The respective magnetic moments of the iron atoms in ferromyoglobin and ferrimyoglobin (horse) are 5.46 and 5.85 Bohr magnetons respectively. The respective molal magnetic susceptibilities are: 12,400 and $14,200 \times 10^{-6}$. These values are essentially the

TABLE I

	pK values for	Heme-Linked Acid Groups	s of Hemoglo	bins	
Compound		pK Values			
Hb+	$pK_1 = 5.3 Mo;$	$pK_2 = 6.65 Si, Mi, Po;$	$pK_3 = 8.10 S$	So, Mo	
Hb	$pK_1 = 5.25 Mi;$	Pi	$pK_2 = 7.81$	Si, Mi	Pi
HbO ₂ HbCO	$pK_1 = 5.75 Mi$,	Po; $pK_2 = 6.80 Si, Mi, Po$, ,	

S=Spectrophotometrically, M=magnetometrically, P=potentiometrically, o=operative, i=inoperative. Since each acid group has a definite effect in any physicochemical equilibrium involving the substance containing it, all are of the class Po, but Pi is used with brackets where cancellation occurs among these in the ferrohemoglobin-ferrihemoglobin electrode potential. (Coryell, C. D., and Pauling, L., J. Biol. Chem., 132, 769 (1940).)

same as the corresponding values for ferrohemoglobin and ferrihemoglobin. Since myoglobin has one or possibly two chemically independent hemes per molecule as contrasted with the four of hemoglobin, the existence of interaction between the hemes of hemoglobin might lead to some difference in the magnetic moments observed for the two substances. However, this is not the case. There appears to be no magnetic interaction between the hemes of hemoglobin (7).

From a consideration of electrode potential and magnetic susceptibility data, Coryell and Pauling (8) have established the existence of an acid group interacting with the heme in ferrihemoglobin. Their pK values of the known acid groups associated with the hemes of various hemoglobins are given in Table I. The dependence of magnetic susceptibility of ferrihemoglobin solutions on pH at various ionic strengths is shown in Fig. 1.

The data given in Table I permit structural interpretations of the

acidity of the heme-linked acid groups in hemoglobin. It is postulated that acid Group I is a histidine imidazolium ion which is in poor position for electrostatic coordination of the basic form with the iron atom. Group II is the imino group of a histidine residue on the opposite side of the porphyrin ring from acid Group I. The 3-nitrogen atom is strongly coordinated by either an essentially

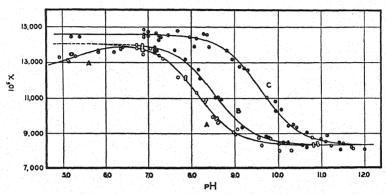
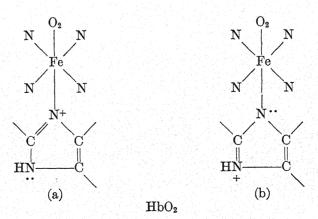


Fig. 1. Dependence of magnetic susceptibility of ferrihemoglobin solutions on pH. Curve A, ionic strength 0.20, pK₁=5.30, pK₃=8.15. Curve B, ionic strength 1.3, pK₃=8.56. Curve C, low and high ionic strength with added fluoride, apparent pK₃=9.62. Molal susceptibilities ($\times 10^{-6}$ c.g.s.u.) Hb⁺I, 12,570; Hb⁺II and Hb⁺III, 14,070; HbF, 14,610; HbOH, 8,340.

(Coryell, C. D., and Pauling, L., J. Biol. Chem., 132, 769 (1940).)

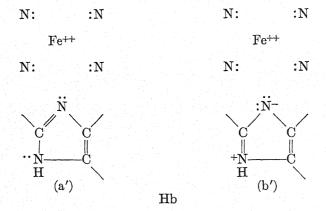
ionic or an essentially covalent bond with the iron atom. Acid Group III of ferrihemoglobin is the iron atom. It may add hydroxide ion, or a water molecule coordinated to the iron atom which may lose a proton.

The resonating forms of oxyhemoglobin may be represented by



If (a) alone were the normal state of oxyhemoglobin, the acidity of the -NH group of the imidazole group would be very low while if (b) were the normal state, the group would be strongly acidic. With resonance between the two structures, the acidity would be intermediate.

The two forms of reduced hemoglobin may be represented by



Structure (b') with separated electric charges is less stable than structure (a') in which the nitrogen atoms have their normal covalence.

The effect of removing the oxygen atom from oxyhemoglobin on the pK_2 value is interpretable from the above structural formulas. Structures (a) and (b) for oxyhemoglobin contribute nearly equally to the normal state of the molecule. Structure (b') of reduced hemoglobin makes a small contribution due to its instability. The normal state must be largely in the form of (a'). Removal of the oxygen atom from oxyhemoglobin leads to a decrease in the acidity of the attached imidazole group.

On the basis of the data presented by Coryell and Pauling, Coryell and Stitt (9) have presented certain revised data for ferrihemoglobin at 25°. The molal paramagnetic susceptibilities (×10⁻⁶) for ferrihemoglobin are: H₂Hb⁺, 12,430; HHb⁺, Hb⁺, 13,910; HbOH, 8,250–8,630. The corresponding magnetic moments are 5.46, 5.77 and 4.45–4.55. Other molal paramagnetic susceptibilities (×10⁻⁶) are: ethanol-ferrihemoglobin ((Hb⁺)EtOH), 14,500; ferrihemoglobin azide (HbN₃), 3,360; ammonia: ferrihemoglobin-hydroxide ((HbOH)NH₃), 3,700; ethanol: ferrihemoglobin-hydroxide ((HbOH)EtOH), 12,150–12,530. The effective magnetic moments corresponding to these compounds are: 5.89, 2.84, 2.98, and 5.39–

5.48. The data indicate that ethanol and ammonia form complexes with ferrihemoglobin. The dissociation constants are: (Hb+)EtOH, 0.4×10^{-6} ; (HbOH)NH₃, 1.0×10^{-6} ; (HbOH)EtOH, 0.39×10^{-6} ; HbN₃, 1.2×10^{-5} ; H₂Hb+, 5.0×10^{-6} .

2. Cytochrome C. Theorell and Åkesson (10) have carried out a study on the properties of the different cytochromes and some of their derivatives in order to compare these with the results obtained by Pauling and coworkers with hemoglobin. Ferricytochrome shows five different spectral forms. The reversible equilibria between them are a function of pH. Form I which exists in strongly acidic solution agrees spectroscopically with "hemin c," the only difference being that in hemin c the peptide linkages binding the protein component to the cysteine residues have been dissolved by hydrolysis. In strongly acid solutions both the hemochromogenforming N atoms are dissociated from the Fe atom. The binding between the Fe+++ atom and the four N atoms in the prophyrin is essentially ionic. Hence the formula may be written as

If the pH of the solution is raised to pH 1.5, Type II form is obtained. Its constitution is essentially similar to that of acid ferrihemoglobin and may be represented by

¹ In this and the other schematic formulas, the four porphyrin nitrogens are written to the right and left of the iron atoms and the two amino acid nitrogens directly above and below.

Ferricytochrome Type III exists in neutral solution. It probably has the mutually resonating constitutions:

The molar magnetic susceptibility, $2,580-3,340\times10^{-6}$, lies close to that for ferrihemoglobin cyanide $(2,610\times10^{-6})$. The iron atom is bound to the surrounding nitrogen atoms by essentially covalent bonds, since it contains only one odd electron.

In Type IV, which exists at about pH 9.6, all six coordination positions of the iron atom are still taken up by nitrogen atoms. The transition from Type III to IV does not imply an addition of hydroxyl ion. Type IV probably has the structures:

The forms without separate electric charges, in which the N atoms of the imidazole groups have their normal covalence resonate with the above forms of Type IV.

Type V, which exists in solutions of very high alkalinity, may be schematically represented by

One of the imidazole groups has been split off from the iron atom.

Cytochrome c has an unusual amino acid makeup which differs markedly from that of hemoglobin or other proteins. It contains about 25 per cent of lysine and 3.3 per cent of histidine. The number of free amino and carboxyl groups suggests that the molecule consists of several polypeptide chains with free amino and carboxyl groups at each end. Two of the three imidazole groups in cytochrome appear to constitute the hemochromogen-forming groups.

There is thus an analogy with hemoglobin which probably contains two imidazole groups in the vicinity of each atom of iron. There is, however, this difference. In hemoglobin, one imidazole group is in a favorable position for coordination with the iron atom and the other group is not. One of the six octahedral valences of the iron atom is left free for the addition of oxygen, carbon monoxide, etc. In cytochrome c, both imidazole groups are in a favorable position for coordination with the iron atom so that a very firm type of compound is formed. However, in strongly acid or alkaline solutions one or both of the imidazole groups are freed from the iron atom with the result that combination with oxygen, carbon monoxide, etc., is now possible.

The heme of the cytochrome is built into the protein moiety by means of thioether linkages from the side chains of the porphyrin to the protein and by means of the two imidazole groups of histidine which are strongly bound to the iron atom on each side of the flat heme disc. It appears then that the heme group is built into a "crevice" of the protein molecule. Since oxygen cannot approach the iron atom, cytochrome c is not autoxidizable. Neither can it form compounds with carbon monoxide or cyanides within physiological pH limits.

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SECTION XII. ANISOTROPY

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Knowledge of the relation of double refraction of flow to molecular size and shape has been much enlarged (27), (28), (29). The most significant quantity to be determined in establishing this relation is the angle, χ , between the optic axis of the flowing liquid and the di-

rection of the stream lines. This is commonly known as the "extinction angle"; it is the complement of the angle of isocline, Ψ , which has already been defined ($\chi+\Psi=90^{\circ}$). (See Fig. 2, page 532.) At very low velocity gradients χ is equal to 45° (or $\pi/4$ radians); as the velocity gradient in the flowing liquid increases, χ decreases, and at very high velocity gradients the optic axis approaches parallelism with the stream lines ($\chi=0^{\circ}$). For a monodisperse solute, in a solvent which shows no double refraction of flow, χ is a continuous function of the parameter $\alpha=G/\Theta$. G is the velocity gradient in the liquid; Θ , the rotary diffusion constant, is a measure of the speed with which the solute molecules, when oriented by an external force, tend to return to a disoriented state, owing to their Brownian movement. It was shown by Boeder (15) that for thin rod-shaped molecules, when χ does not differ from 45° by more than a few degrees, the relation between χ and α is (χ is expressed in radians):

$$\chi = \frac{1}{2} \tan^{-1} \frac{6}{\alpha} = \frac{\pi}{4} - \frac{\alpha}{12} \left(1 - \frac{\alpha^2}{108} + \cdots \right)$$
 (1)

For ellipsoids of revolution, with semi-axis of revolution a, and equatorial semi-axis b, Peterlin and Stuart (28) obtain the relation

$$\chi = \frac{\pi}{4} - \frac{\alpha}{12} \left[1 - \frac{\alpha^2}{108} \left(1 + \frac{24}{35} \frac{a^2 - b^2}{a^2 + b^2} \right) + \cdots \right]$$
 (2)

For small α values, equations (1) and (2) are practically identical. Thus the determination of a value of χ in the flowing liquid, if χ is near $\pi/4$ radians, fixes the value of $\alpha = G/\Theta$. G is known from the dimensions of the concentric cylinder apparatus and the speed of the rotating cylinder. If the radius of the inner cylinder is R_1 , that of the outer cylinder R_2 , and if $(R_2 - R_1) \ll R_1$, then to a close approximation:

 $G = \frac{R_1 \Omega}{R_2 - R_1} \tag{3}$

where Ω is the angular velocity of the rotating cylinder. Thus from the definition of α , and equations (1) and (3), the rotary diffusion constant, Θ , is fixed by a measurement of χ (or of Ψ).

These relations hold only when the solution is so dilute that χ , for a given velocity gradient, is independent of the concentration of solute. At higher concentrations the particles may not orient independently, especially if they are very elongated, and the value of χ may be greatly affected by their interaction.

 Θ is proportional to the reciprocal of the relaxation time, τ ,

which is discussed in the Addendum to Chapter XVI. The relation of Θ and τ to the size and the shape of particles, which may be regarded as ellipsoids of revolution, has been given by Perrin (30). An ellipsoid of revolution has two rotary diffusion constants, corresponding to motion about the a-axis and about the b-axis, and two corresponding relaxation times. Perrin's general equations are somewhat complex, but for an elongated ellipsoid in which the axial ratio a/b is greater than five, the value of the smaller rotary diffusion constant—which is the one determined from equation (1) or (2)—is given quite accurately by the relation:

$$\Theta = \frac{1}{2\tau} = \frac{3KT}{16\pi\eta a^3} \left[2 \ln \frac{2a}{b} - 1 \right]$$
 (4)

Here K is Boltzmann's constant, T the absolute temperature, and η the viscosity of the solvent. a and b have been defined above (see equation (2)). The value of Θ depends almost entirely on the value of the major semi-axis a, being nearly inversely proportional to a^3 . Values of a/b to be employed in the term in brackets in (4) may be derived from viscosity measurements (30a) but the exact value of a/b employed makes little difference in the calculated value of the length, 2a, which may be derived from the rotary diffusion constant by equation (4).

For a very flattened disc-shaped ellipsoid ($a \ll b$), the two rotary diffusion constants and relaxation times become identical, and are given by the relation:

$$\Theta = \frac{1}{2\tau} = \frac{3KT}{32\eta b^3} \tag{5}$$

In this case Θ depends only on the inverse cube of the b semi-axis, and is independent of the a semi-axis.

In order to obtain orientation of molecules that are not very asymmetrical, high velocity gradients are required. This involves making the gap between the concentric cylinders very narrow. A much improved design for the optical system required to make accurate measurements under such conditions has been described (29), (31); also the construction of the concentric cylinder apparatus itself has been much improved and the conditions required for the maintenance of laminar flow in the liquid have been carefully considered (32), (33).

In polydisperse solutions the relations between χ and the velocity gradient become much more complex than if the solute is mono-

disperse (34), (29). Under certain conditions, x may increase, instead of decreasing, with G over a certain range; if two components of different size and shape are present, one giving positive, the other negative, double refraction, x may actually shift across the stream lines, going from positive to negative values, as the velocity gradient increases. This has been shown experimentally for a solution containing methyl cellulose and sodium thymonucleate (34). Somewhat similar curves are shown (35) by serum globulin dissolved in a glycerol-water mixture. After extraction of the globulin with ether and acetone at 0°, the anomaly disappears. and the system behaves like a single component. Presumably a lipid. of negative birefringence, is removed by the extraction, leaving the positive protein component to exert its influence alone. Serum globulin in water also behaves like a single component; here presumably the protein and lipid are firmly united. Addition of glycerol breaks up the combination.

The rotary diffusion constants, and estimated lengths, of certain proteins, are given in Table I, taken from a recent general review of this field (40). The lengths of two specific antipneumococcus polysaccharides, S_I and S_{III} of Heidelberger, have been estimated as 1,560 and 3,300 Å, respectively (29). Tobacco mosaic virus has been studied by several workers (37), (39), (39a), (41), (42); the estimate of the length, given in Table I, is in satisfactory agreement (considering the tendency of the virus particles to aggregate, and the wide differences between different preparations) with the length of about 3,000 Å for the fundamental unit, obtained directly from electron microscopy (43), (44).

The intense positive double refraction of flow in myosin is profoundly diminished in a few seconds or minutes by many relatively mild reagents. Among these are guanidinium salts, chlorides of calcium, magnesium, and barium, and (at higher concentrations) lithium and ammonium salts, in addition to the reagents mentioned on page 533. Evidently these reagents break up the myosin into smaller and less asymmetrical particles. Loss of double refraction is invariably associated with decrease of viscosity (45) since both properties depend on the asymmetry of the molecule, but there is no correlation of either with changes in titratable —SH groups (46). Sodium thymonucleate (47) shows intense negative double refraction of flow, which is profoundly diminished by addition of a number of salts. Guanidinium halides, especially the iodide, were found to be especially powerful in their action. Re-

			TABLE I			
Re	ntary Diffusion	n Constants a	nd Approximate Lengths	of Certain	$Proteins\ and$	of
			Sodium Thymonucleate			

Substance	Ref.	Temp. °C	Θ(sec. ⁻¹)	Length, 2a
Myosin (rabbit)	(9)	3	7	11,600 Å
Myosin (snail)	(36)	25	ca. 1	ca. 28,000 Å
Myosin (octopus)	(36)	25	3.5	18,000 Å
Tobacco mosaic virus				
pH 6.8	(37)	3	25	7,200 Å
Tobacco mosaic virus,				
pH 4.5	(37)	3	0.75	24,000 Å
Sodium caseinate				
(in 1.6 N Na ₂ SO ₄)	(33)	20	700	2,200 Å
Hemocyanin (Helix)	(29)			890–960 Å
Horse antibody globulin				
(mol. wt. 990,000)	(29)			1,280 Å
Sodium thymonucleate	(39) (39a)	20 (?)	180	4,500 Å

The values of Θ are calculated from equation (1); those of the length 2a from equation (4). The lengths given here are not always those given in the original communications. Mehl (36, 37) and Nitschmann and Guggisberg (33) have used the equation of Werner Kuhn (16) for the length S:

$$\Theta = \frac{8KT}{\pi \eta S^3}$$

The results given here, however, are all still attended with some uncertainty. The value of a/b was taken as 100 for myosin, and for tobacco virus at pH 4.5, in applying equation (4); it was taken as 50 for tobacco virus at pH 6.8 and as 5.8 for Na caseinate in 1.6 N Na₂SO₄, the latter being the value estimated (33) from viscosity measurements. The value of a/b was chosen as 200 for sodium thymonucleate.

The sodium case in ate solution in 1.6 N Na₂SO₄ was certainly highly aggregated; many of the case in solutions studied by Nitschmann and Guggisberg (33) consisted of much smaller particles.

Tobacco mosaic virus has also been studied by Kausche, Guggisberg, and Wissler (39) and by Robinson (42), with results in generally good agreement with those of Mehl (37).

The double refraction of all protein solutions yet studied is positive; that of sodium thymonucleate is negative.

(Edsall, J. T., in "Advances in Colloid Science." Edited by E. O. Kraemer, Interscience Publishers, New York, Vol. I, p. 310, 1942.)

moval of the salt, however, almost completely restored the double refraction in sodium thymonucleate solutions, while in myosin the effect of denaturing agents was apparently irreversible (see however (48)).

The spontaneous aggregation and orientation of anisotropic particles gives rise to remarkable phenomena in tobacco mosaic virus (49). Virus solutions of medium strength divide into two

layers on standing. The top layer which is more dilute—virus concentration 1.6 to 4 per cent— is an isotropic liquid showing double refraction of flow (see Table I). The bottom layer, and the higher concentrates produced by centrifuging, are spontaneously doubly refractive and consist of regions in which the particles are parallel. X-ray diffraction studies show that the particles, viewed in a plane at right angles to their long axis, form a new type of liquid crystal, with a regular two dimensional hexagonal pattern. The interparticle distance varies with concentration, but the nature of the hexagonal pattern is the same at all concentrations, from a dry gel of virus, down to a 13 per cent solution in water, and probably even in solutions much more dilute than this. There is no regularity in the arrangement of the particles, however, in the direction parallel to their length. In an air dried gel, the distance between the centers of adjoining particles is 152 Å; this figure may be taken as the diameter of the particles; it is confirmed by studies with the electron microscope (43), (44). In a 13 per cent solution, the particles are about 400 Å apart, yet their parallel orientation and hexagonal arrangement are apparently maintained perfectly. This orientation is probably determined by interionic forces, arising from the charge on the virus particles themselves and from the smaller ions surrounding them in the aqueous solution (50). The principles involved are quite general, and should apply to other highly asymmetrical molecules that carry a large electric charge. They may explain many orientation phenomena in colloidal and biological systems.

The virus particles themselves have an internal regularity of structure, like that of a crystal, as shown by the x-ray studies. The same is also true of the virus particles of tomato bushy stunt disease. The latter, however, are nearly spherical (49) showing that viruses are not necessarily characterized by an elongated shape.

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CHAPTER X

OPTICAL PROPERTIES OF AMINO ACIDS AND PROTEINS

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1. Optical Rotation of Amino Acids. Theories of optical rotation which are based on quantum mechanics have been developed in recent years. These account for the observed orders of magnitude of the optical rotations and show how the rotatory power depends on molecular structure (see (54)). Two influences are involved in making a molecule capable of rotating the plane of polarization of plane polarized light. The first of these is the structural one. This permits the molecule to exist in non-superimposable mirror image forms (enantiomorphs). The asymmetric carbon atom is only one of the possible factors in a molecule that may operate to give rise to non-superimposable mirror-image forms. The second influence, which is the more complex, and less understood, is the mechanism that makes possible the interaction of a given enantiomorphic form of the molecule with a plane polarized light wave so as to rotate its plane of polarization. Approximate solutions of the nature of the interaction have been obtained by the application of quantum mechanics.

The recent developments on the configurational relationships of the amino acids and a desirable terminology resulting therefrom are discussed by Dunn in the Addendum to Chapter II. The configurational relationships among the amino acids have been derived, to a considerable extent, from the optical displacement rule proposed by Freudenberg as a basis for determining the relative configurations of similar substances whose relationship could not be established in any other way. This rule states that differences in rotation between analogous derivatives of analogously constructed compounds are approximately the same in magnitude and direction (55). Obviously this rule cannot be applied to too structurally diverse sets of compounds. The application of this rule to the configurational relationship between alanine and lactic acid is illustrated in Fig. 1,

which shows that the rotations of analogous derivatives of the two compounds are approximately the same in magnitude and direction.

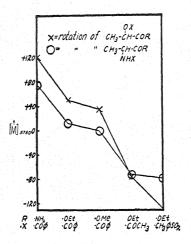


Fig. 1. Comparison of the optical rotation of analogous derivatives of lactic acid and of alanine.

(Kauzmann, W. J., Walter, J. E., and Eyring, H., Chem. Rev., 26, 339 (1940). Courtesy of the Williams and Wilkins Company.)

In the change of reaction from acid to basic solutions, amino acids go through the transformations,

Since the groups represented by R in the amino acids are all fairly similar, according to the optical displacement rule regularities should be observed in the rotations which accompany these changes, providing all amino acids possess the same stereochemical configuration. That this is the case has been shown by Lutz and Jirgensons (see Chapter X, page 578). Kauzmann, Walter, and Eyring (54) account this as the best sort of evidence that the naturally occurring amino acids possess the same configuration about their α -carbon atoms.

A new table of data on the specific rotations of the accepted amino acids and of reported amino acids, supplementing Table V of Chapter X and containing recent measurements is included here (Table I).

Table I

(a) Specific Rotations of the Accepted Amino Acids*.1

		S-1			Moles aci or base/		Temp.		., 1	Ref.
Source) C	Solvent	d	p	mole amino aci	L	°C	αр	OV D	no.
				l(+)-Ala	nine	100				7
A	5.790	0.97 N HCl	1.033	5.605	1.5	2	15	+1.70	+14.7	1
A	10.3	Water	1.03	1.00	0	2	22	+0.55	+2.7	2
\overline{A}	1.781	3 N NaOH			15	2	20	+0.11	+ 3.0	3
	202	0 11 110011		d(-)-A		-	-0	10.11	, 0.0	ŭ
В	1.344	6 N HCl		w()-11	39.4	2	30.4	-0.392	-14.6	4
ь	1.044	O IN IICI		7/ 1 \ 1.		2	30.4	-0.592	-14.0	**
	1 050	a a at trai		l(+)-A			00.4		1.00.0	5
\boldsymbol{A}	1.653	6.0 N HCl			63	4.001	23.4	+1.777	+26.9	
A	3.48	Water			0	2	20	+0.87	+12.5	6
\boldsymbol{A}	0.87	0.50 N NaOH	4.5		10	2	20	+0.22	+11.8	6
			l(+)-Aspa	artic acid					
A	2.002	6.0 N HCl			39	4.001	24.0	+1.972	+24.6	7
\boldsymbol{A}	1.3300	Water			0	3	18	+0.19	+4.7	3
\boldsymbol{A}	1.3300	3 N NaOH			30	3	18	-0.068	- 1.7	3
			d	(-)-Asp	artic acid	1				
C	4.289	0.97 N HCl	1.032	4.156	3	1	20	-1.09	-25.5	8
				l(-)-C						
\boldsymbol{A}	0.9974	1.02 N HCl	1 0181	0.9797		2	24.35	-4.277	-214.40	9
A	0.400	0.20 N NaOH	1.0101	0.0101	12	2	18.5	-0.56	-70.0	3
A.	0.400	0.20 N NaOII		4/ 13 (-	10.0	-0.00	-1,0.0	٠,٠
~		* 37 TTCI		d(+)-(00		1 000	10
C		1 N HCl		1	24		20		+223	10
					otyrosine		122			
A	5.08	1.1 N HCl	1.05	4.84	9.4		20	+0.15	+2.89	11
A	4.41	13.4 N NH ₄ OH	0.9779	4.51	132	1	20	+0.10	+2.27	11
			l(+)-Glut	tamic aci	d				
A	1.002	6.0 N HCl			87	4.001	22.4	+1.25	+31.2	12
A	1.471	Water			0	2	18	+0.34	+11.5	3
A	1.471	1 N NaOH			10	2	18	+0.309	+10.96	3
			d	—)-Glu	tamic ac	id		A Section		
C	5.425	0.37 N HCl	1.0233			1	20	-1.63	-30.05	8
Ŭ	0.120	0.01 2201			Histidine		-75,431			
A	1.480	6.0 N HCl			63	4.001	22.7	+0.766	+13.0	7
			1.0012	1 197	0	4	25.00	-1.714	-39.01	18
A	1.128	Water	1.0012	1.127				-0.169	-10.9	10
\boldsymbol{A}	0.775	0.50 N NaOH		77.13.7	10	2	20	-0.109	-10.9	
				a(+)-1	Iistidine		20	0 10	10.0	
?	4.000	1.0 N HCl			4	1	20	-0.407	-10.2	1
B	2.66	Water			0	2	23	+2.11	+39.8	1
			l(十)・	Hydrox	yglutami					1
A	1.33	6.0 N HCl			73	2.0	20	+0.47	+17.6	. 1
A	4.0	Water			0	2.0	20	+0.10	+ 1.2	1
			l	(-)-Hy	droxyprol	ine (a)5				
A	1.31	1.0 N HCl			10	2	20	-1.24	-47.3	1
A	1.001	Water			0	4.00		-3.009	-75.2	100
A	0.655	0.50 N NaOH			10	2	20	-0.925	-70.6	
	0.000	0.001414011	26	\ Hada	oxyprolin			0.020		
	4.40	YY7			oxyproiii O	1 (a)	21	+3.37	+75.2	1
В	4.48	Water	1.03	4.35			41	₩75.51	₹10.2	
15.2					oxyproli				FO 4	
В	2.617	Water	1.014	2.58		1	18	-1.52	-58.1	1
				-)-Hydro	xyprolin	e (b)				
B	2.530	Water	1.013	2.998	8 0	1	17	+1.48	+58.5	1
				l(+)-I	soleucine					
В	5.09	6.1 N HCl	1.098	4.64	15	1	20	+2.07	+40.61	1
В		Water	1.008		0	2	20	+0.70	+11.29) 1
A		0.33 N NaOH	1.017	3.28			20	+0.74	+11.09	
А	0.04	0.00 IV IVAUII	1.011		-Isoleucii		~~~	, ,,, ,		
_		a 1 NI IICI	1 000				20	-1.85	-40.86	3 :
В		6.1 N HCl	1.083			1				
В	3.12	Water	1.006	3.10	0	2	20	-0.66	-10.58	٠.,

TABLE I—Continued

					Moles acid	i				
Sourc	e c	Solvent	d	p	or base/ mole	ı	Temp. °C.	αD	[α] _D	Ref
					amino acid	i		<u> </u>		
			d(-)-AU	o-Isoleucine					
D	5.14	6.0 N HCl	1.094	4.70	15.0	2	20	-3.80	-36.95	19
В	2.00	Water			0	1	20	-0.285	-14.2	20
			l(+)-Allo	-Isoleucine					
В	3.97	6.0 N HCl			20	1	20	+1.50	+38.1	20
В	2.00	Water			0	1	20	+0.28	+14.0	20
	1 000	0.037.7701		l(—)-	Leucine	4 001	25.9	11 919	118 1	5
A A	1.999 2.001	6.0 N HCl Water			38 0	4.001	24.7	+1.212 -0.863	+15.1 -10.8	5
A	1.31	3.00 N NaOH			30	2	20	+0.20	+7.6	3
	1.01	0.0011114011		d(+)-	Leucine			, 0		į
?	4.0	6.0 N HCl	1.1	3.664		2	20	+1.26	-15.6	21
?		Water		2.08	0	2	20	+0.43	+10.34	38
				l(+)	-Lysine					
A	2.00	6.0 N HCl			43	4	22.9	+1.652	+25.9	5
A	6.496	Water			0	2	20	+1.90	+14.6	22
				d(-)-						
В	2.00	0.27 N HCl	Carlotte,		2	2	20	-0.939	-23.48	23
_			l	(–)-M	lethionine	_				٠.
В	0.80	Water			. 0	2	25	-0.13	- 8.11	24
В	0.00	0 0001 N HCI	o	((+)-IV	Iethionine	0	0.5	0.94	01 10	0.4
В	0.80	0.2001 N HCl Water			4 0	2	25 25	-0.34 + 0.13	-21.18 + 8.12	
В	0.80	0.6 N NaHCO:			11	2	25	-0.13	-7.47	
	0.00	0.6 N Nameor		/(±1-N	orleucine	-	20	-0.12	- 7.27	24
В	4.25	6.0 N HCl	1.10		18	2	20	+1.81	+21.3	25
В	0.75	Water	1.00	0.753		2	20	+0.095	+ 6.26	
					Torleucine					
B	4.69	6.0 N HCl	1.10	4.26	16	2	20	-2.10	-22.4	25
В	0.96	Water	1.00	0.959	0	2	20	-0.087	- 4.49	26
jin.			l(enylalanine					
В	1.936	Water	1.0040	1.928		2	20	-1.36	-35.14	27
_					enylalanine	<u>.</u> 16 (4)				
B	3.814	5.4 N HCI	1.0895			2	20	+0.54	+ 7.07	
В	2.043	Water	1.0045	2.034		2	20	+1.43	+35.0	27
A	0.575	0.50 N HCl		i(-)-	Proline 10	2	20	0 605	E0 6	6
A	1.001	Water			0	4.001	23.4	-0.605 -3.402	-52.6 -85.0	7
В	2.42	0.6 N KOH	1.031	2.35	3	1	20.4	-2.25	-93.0	29
					-Proline	7.356	155			
В	3.90	Water	1.01	3.865		1	20	+3.18	+81.5	29
				l(-)	-Serine					
В	9.344	1 N HCl	1.0465	8.929) 1	1	25	+1.35	+14.45	30
В	10.414	Water	1.0414	9.997	0	2	20	-1.42	- 6.83	30
	Grant at Elever				-Serine					
B	9.359	1 N HCl	1.0465	8.943		1	25	-1.34	-14.32	
B	10.412	Water	1.0414			2	20	+1.43	+6.87	30
В		TYP 4			hreonine	_				
ь		Water		1.092		2	26	-0.625	-28.3	31
В		Water		100	hreonine	2	26	±0.780	L98 4	91
Ĩ.,		Water	20	1.331	l 0 5-Threonine		26	+0.780	+28.4	31
В		Water		1.634		2	26	-0.302	- 9.1	31
J.			?(-Threonine		40	0.002	9.1	OI
В		Water		1.643		2	26	+0.320	+ 9.6	31
					hyroxine					7
A		0.13 N NaOH		3	3	1		-0.147	- 4.4	32
		in 70 per cent								
		EtOH by weight								

TABLE I-Continued

					Moles acid					
Source	c	Solvent	đ	p	or base/ mole amino acid	l	Temp. °C.	αD	[α]D	Ref.
			2.(—)-Trvi	ptophane					
\boldsymbol{A}	1.02	0.50 N HCl	7.	,		2	20	+0.049	+2.4	6
\boldsymbol{A}	1.004	Water			0	4.001	22.7	-1.266	-31.5	7
$^{\circ}A$	2.426	0.5 N NaOH	1.0243	2.368	4.2	1	20	+0.15	+6.17	33
			d(+)-Try	ptophane					
C	0.5024	Water			0	2	25	+0.326	+32.45	34
				l(-)-T						
\boldsymbol{B}	4.40	6.3 N HCl	1.116	3.94	28	2	20	-0.76	- 8.64	
A	0.906	3.0 N NaOH			60	3	18	-0.359	-13.2	3
112				d(+)-T						
B	5.1484	6.3 N HCl	1.1175	4.6071		2	20	+0.89	+ 8.64	35
		4 4 N T TTC!			Valine		00	11.00	100.0	00
B	3.4	6.0 N HCl	1.1	3.05	20	2	20	+1.93	+28.8	36 36
В	3.58	Water	1.007	3.56	0	2	20	+0.46	+6.42	30
		6.0 N HCl			Valine	2	20	-1.86	-29.04	36
В	3.2		1.1	$\frac{2.91}{6.24}$	21 0	1	20	-0.37	-6.06	
\boldsymbol{E}	6.24	Water	1.00	0.24	U	1	20	-0.37	- 0.00	31
	(7.)	g . : (D		n.		77.				
	(6)	Specific Rotations of	Amino A	icias Ke	portea out	not ve	rinea an	a oj Aspara	gine	
			l(+)-a	-Amino	-n-butyric	acid				
В	3.746	0.36 N HCl	1.0201	3.672	1.00	2	20	+1.46	+19.49	
\boldsymbol{B}	5.461	Water	1.0102	5.406	0	2	20	+0.87	+ 8.0	28
			d(-)- a	-Amino	-n-butyric	acid				
\boldsymbol{B}	3.594	0.35 N HCl	1.0202	3.523	1.00		20	-1.40	-19.48	
В	5.36	Water	1.009	5.31	0	2	20	-0.85	-7.92	28
			Asparagi	ne(l(+	-)-Asparta	mide)				
A	9.517	10 per cent HCl			4.2		20		+28.6	43
A	1.41	Water	100	1.1	0	2	20		- 5.6	44
?	12.46	1 N NaOH	1.062	11.73	1.0	3	25	-3.50	- 9.36	3 45
					aline ⁶		0.4		- 8.1	39
		Water		1.6	11: 6		21		- 0.1	08
	4 054	0.3 N HCl		Citr	ulline ⁶ 1	2.2	22	+1.96	+17.9	49
A	4.974	Water			Ô	2.2	22	+0.38	+ 3.4	
A	5.005	3 N NaOH		5	10	2.2	20	10.00	+ 8.8	
\boldsymbol{A}		5 IV IVROIT			Cysteine		-~			
A	2.099	1 N HCl		•()-(56.6	4.001	22.9	+0.605	+ 7.2	1 7
A	2.420	Water			0	4.001		-1.002	-10.4	
A	2.720	77 2001	1(-)-3.4	-Dihvdi	roxyphenyl					
A		1 N HCl	-()-1-	9.81	2.01		20	-1.40	-14.2	8 40
A	0.493	Water			0.0	2	20	-0.33	-33.3	•
	0.100			?(-)-I	sovaline2					
D	4.02	20 per cent HCl	1.0943		16	2	20	-0.48	- 6.1	1 46
D	5	Water			0	2	20	-0.91	- 9.1	0 4
-				l(?)-N	orvaline					
A		20 per cent HCl		5	12		20	+1.14	+22.8	4
				1(+)-0	rnithine					
\boldsymbol{A}	6.83	1.03 N HCl			2	2.2	25	+4.01	+26.7	
A		Water		6.5	0		25		+11.5	
	0.661	0.50 N NaOH3			10		20		+ 7.0	
A										
A			l	(?)-2 - Th	iolhistidin	e 0.5			- 9.5	4 4

^{*} The data contained in this table were kindly compiled by Dr. M. S. Dunn with the assistance of Mr. M. P. Stoddard.

¹ The specific rotations are given in all cases for the free amino acid. The values quoted are those considered to be the most reliable. In some cases the specific rotations listed were calculated from the author's values for the anhydrous or hydrated hydrochlorides. Some of the values for c, d, p, α , normality of the acid

TABLE I-Continued

or base, and molar ratios of acid or base to amino acid were calculated from data reported in the papers cited in the bibliography. In a recent discussion of the criteria for acceptance of an amino acid Vickery (51) lists 25 amino acids that he consideres to be constituents of proteins.

- c-gm. of solute per 100 ml. of solution.
- d-density of the solution.
- p-gm. of solute per 100 gm. of solution.
- l-length of the tube in decimeters.
- a-observed rotation in angular degrees.
- [α]-specific rotation in angular degrees calculated from

$$[\alpha]_{\lambda}^{t} = \frac{[\alpha] \times 100}{c \times l} = \frac{\alpha \times 100}{p \times d \times l}$$
 where t is °C. and λ is wave length of the incident light in Ångström units.

 $[\alpha]_D = [\alpha]_{5893}.$

- A-prepared from a protein or other naturally occurring material.
- B-prepared by resolution of the inactive synthetic form.
- C-prepared by resolution of the inactive racemized form.
- D-prepared from the inactive synthetic form by a biological method.
- E-prepared from the inactive racemized form by a biological method.
- ?-unknown.
- ² The configuration is uncertain since (a) the authors (46) prepared the amino acid by the action of yeast on the dL-(synthetic)-form and (b) the specific rotation is more positive in acid than in water. The unnatural (d) antipode would result normally from (a) while, according to the rule of Lutz and Jirgensons (3,6), the evidence given in (b) would point to the natural (l)-form.
 - 3 In a solution containing two moles of sodium chloride per mole of free base ornithine.
 - ${}^{4}\left[\alpha\right]_{\lambda}=[\alpha]_{5:161}.$
- ⁵ The stereoisomeric hydroxyprolines are referred to as (a) and (b). Type (a) includes 2 optical isomers which are designated as l(-) and d(+), the former occurring in proteins. The (b) type also includes 2 optical isomers, both unnatural. There is no evidence as to which of these isomers corresponds to the d- and which to the l-form. Therefore only the direction of rotation in water is indicated. The term allo-hydroxy-proline more appropriately designates the members of type (b).
 - 6 Complete data are not available.

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- 2. Rotatory Dispersion. Carpenter and Lovelace (56) observed that concentrated urea lowers the rotatory disperson of gelatin in the same manner as the alkali halides (Chapter X, page 592). The maximum lowering of the rotatory dispersion constant of gelatin by urea is about two-thirds of the lowering produced by the sodium halides.

Sata (57) found that the optical rotation of gelatin was decreased by ultrasonic waves. Short exposures of 1 to 7 minutes only liquefy the gel structure. The optical rotation, like the thixotropic phenomenon, is reversible. Long exposures produce a degradation of the protein molecule in which the optical rotation remains permanently altered.

3. Raman Spectra. A comprehensive summary of the deductions regarding the structure of the amino acids and related compounds obtained from the study of the Raman spectra has been published by Edsall (58). The principle Raman frequencies of the different ionic forms of glycine and alanine and of related fatty acids are shown in Fig. 1, Chapter XVI, page 876. Fig. 2 shows a plot of the Raman spectra of the hydrochlorides of amino-n-butyric, amino-isobutyric, amino-n-caproic, leucine, and isoleucine. Edsall's results have been verified and extended by Ananthakrishnan (59), Goubeau and Lüning (60), and by Wright and Lee (61). Ananthakrishnan measured the Raman spectrum of crystalline glycine. Goubeau and Lüning examined the spectra of glycine and also of gelatin in solutions varying in pH from 0 to 13. Wright and Lee

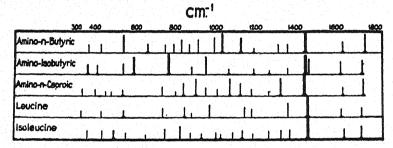


Fig. 2. Raman spectra of two α -amino-butyric acids and three α -amino caproic acids, in the form of their hydrochlorides.

(Edsall, J. T., Cold Spring Harbor Symposia Quant. Biol., 6, 40 (1938).)

have succeeded in obtaining a large number of the Raman lines of isoelectric valine, leucine, and phenylalanine in dilute aqueous solution.

A rule that has proved important in the assignment of Raman spectral bands is that only molecules or groups bound by covalent linkages give rise to Raman frequencies in the range subject to examination.

Proof of the dipolar ion structure of amino acids (see Chapter XVI, page 876) is that the frequency characteristic of the carbonyl portions of the carboxyl groups is shown only by the hydrochlorides of the amino acids and not by the neutral amino acids. The hydrochlorides of the dicarboxylic amino acids, aspartic and glutamic acids, show intense Raman lines near 1740 cm⁻¹ as would be expected. The mono-sodium salts of these amino acids do not exhibit

lines of this frequency. The ionized carboxyl group gives rise to strong Raman frequencies near 1400 cm⁻¹, but these are more difficult to recognize since they may be masked by coincident lines arising from a methyl or methylene group.

Un-ionized primary amines show two powerful frequencies, the lower and more intense at about 3310 cm⁻¹, the other at 3370 cm⁻¹. Glycine ethyl ester shows amino group frequencies at 3328 and 3408 cm⁻¹. On ionization of the amino group, the frequencies above 3300 cm⁻¹ disappear and are replaced by much broader and weaker frequencies in the region 2500 and 3200 cm⁻¹. Solutions of sodium glycinate and sodium alaninate show strong frequencies characteristic of the neutral amino group near 3320 cm⁻¹, in complete contrast to the isoelectric amino acids that do not. Certain other characteristic frequencies of compounds containing —NH₂ and —NH₃+ groups have been more difficult to identify because of overlapping by the lines of other groups.

To identify the several frequencies that were uncertain, Edsall and Scheinberg (62) studied the spectra of deutero substitution products of methylamine and hydrazine. This investigation showed that the $-NH_2$ and $-NH_3$ + group frequencies are higher by a factor of 1.33 to 1.36 than in the corresponding deutero compounds. For $-ND_3$ +, the principal valence vibration lies near 2180 and in $-NH_3$ + near 2970 cm⁻¹. The principal deformation frequency of $-NH_3$ + and $-NH_2$ groups is near 1620, of the corresponding $-ND_3$ + and ND_2 groups near 1200 cm⁻¹.

The Raman spectra of urea and of the guanidinium ion which are of interest in connection with the structure of the amino acids citrulline and arginine are quite similar, both having a very intense frequency at almost exactly 1000 cm⁻¹. The spectrum of the guanidinium ion, however, is distinctly simpler than that of urea, indicating that it has a higher degree of symmetry than urea. Besides the line mentioned above, urea has lines at 1170, 584, and 521; guanidinium ion has a line at 533. The Raman spectra are in harmony with the classical carbamide formula for urea¹ and a structure for the guanidinium ion in which the three C-N bonds are entirely symmetrical and equivalent. The energies of these bonds correspond more nearly to those of double than to single bonds.

¹ In this connection see Kumler, W. D., and Fohlen, G. M., J. Amer. Chem. Soc., 64, 1044 (1042).

This would lead to the formula given below for the guanidinium ion:

The Raman spectra can also be used as a mode of differentiating between closely related amino acid isomers, because the molecular vibrations are extremely sensitive to small differences in configuration. This is illustrated by the profund difference in the spectra of the hydrochlorides of amino-n-butyric and amino-isobutyric acids, of leucine, and of isoleucine as shown in Fig. 2.

4. Absorption Spectra. Magill, Steiger, and Allen (63) have measured the ultraviolet absorption spectra of a large number of amino acids, peptides, and their acetyl, benzoyl, and benzenesulphonyl derivatives. The data are too extensive to be reproduced here and the reader is referred to the original for the details. The significant conclusions from this work are that the absorption of light by a dipeptide is greater than that of either or both of its constituent amino acids. Substitution of a hydrogen of the amino or imino group by either an acetyl, benzoyl, or benzenesulphonyl group, displaces the absorption curve to regions of longer wavelengths.

Proteins exhibit selective absorption in the ultraviolet region of the spectrum with a maximum at about 2800 and a minimum at about 2500 Å, which has been interpreted as being caused by the aromatic groups of the amino acid constituents, tryptophane and tyrosine (see Chapter X, page 555). Anslow and coworkers (64, 65) state that the peptide linkage is responsible for a region of selective absorption at about 2800 Å which is common to proteins and simple peptides. This absorption band was observed in glutathione and gelatin, which are lacking in the aromatic amino acid residues. The intensity of the absorption is low as compared to that of proteins containing the aromatic amino acids. The band due to the peptide linkage is shifted toward the visible region in alkaline and alcohol solutions. Representative absorption curves showing the characteristics of the absorption by the peptide linkage are shown in Fig. 3.

From the mean wave-length of the peptide bands in aqueous solutions, it was calculated that 4.4 electron volts of energy are

absorbed in breaking this linkage. Alcohol and alkali decrease the required energy.

Absorption spectra of many and varied proteins have recently been reported. These include the enzymes papain (66) and crystalline ribonuclease (67), vitellin from the egg yolks of the hen and quail (68), and from the egg yolk of loggerhead turtle (69), wheat glutenin (70), the soy bean proteins (71), and various purified plant viruses (72–74). Except for the viruses, the absorption spectra

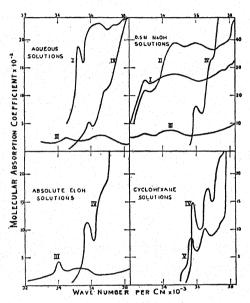


Fig. 3. Ultraviolet absorption curves of compounds containing the peptide linkage. Absorption curves of I. gelatin; II. lactalbumin; III. glutathione; IV. acetanilide; V. stearic anilide. The NaOH curves are plotted at half the scale of the others and lactalbumin values are reduced one-third.

(Anslow, G. A., and Nassar, S. C., J. Opt. Soc. Amer., 31, 118 (1941).)

of the proteins are largely determined by their content of the amino acid residues of tyrosine and tryptophane. The purified plant viruses exhibit absorption maxima in the region of 2650 Å. This band is characteristic of the purine and pyrimidine components of nucleic acid.

5. Infra Red Spectra. The evidence derived from the infra-red spectrum of gelatin regarding the hydration of proteins has been discussed in the Addendum to Chapter IX, Section III. In a recent study, Bath and Ellis (75) determined the near infra red absorption

spectra of silk fibroin and crystalline zinc insulinate as well as of gelatin. A diagram showing the location of the principle absorption maxima of the above proteins is given in Fig. 4.

The infra red absorption spectra of the proteins can readily be interpreted in terms of the overtone valence vibrations and combinations of the fundamental valence vibrations and deformation vibrations of CH, NH, and C=O groups in the protein molecules. The terms due to the different groups are so labeled in Fig. 4.

With an unpolarized source of light, the weak absorption band for C = O (at 1.90μ), it will be observed, was visible only in the silk fibroin spectrum. When plane polarized light was employed, the absorption band at 1.90μ became much stronger when the light

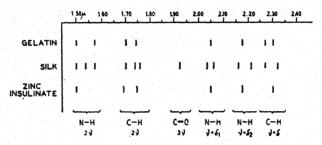


Fig. 4. Diagram showing location of absorption maxima obtained from gelatin, silk fibroin, and zinc insulinate in the infra-red region of 1 to 2.5μ. (Bath, J. D., and Ellis, J. W., J. Phys. Chem., 45, 204 (1941).)

was vibrating perpendicular to the fiber axis in the case of silk fibroin. This indicates that the majority of the C=0 bonds lie in the plane perpendicular to the axis of the silk fibroin strands.

6. Fluorescence. Reeder and Nelson (76) have observed that proteins irradiated with ultraviolet light of 3100-4100 Å give a uniform bluish-white fluorescence in the solid state and a greenish fluorescence in solution, which is stronger in alkaline solution. The fluorescence is destroyed by oxidation with HNO₃ or ashing.

None of the known amino acids exhibit fluorescence in the solid state or in solution. Nineteen amino acids were tested and, from structural consideration, it seems highly improbable that those unavailable for test would do so.

Boiling the proteins with acetic acid greatly increased the amount of fluorescence. Tyrosine and tryptophane, when boiled in dilute HCl in the presence of glucose, gave blue-green fluoresence.

The fluorescent material in the protein appears to be a condensation product of tryptophane and tyrosine. On concentration by adsorption, it gave a fluorescent spectrum in the violet-green region, similar to the fluorescent spectrum of quinine bisulfate.

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CHAPTER XI

AMPHOTERIC PROPERTIES OF AMINO ACIDS AND PROTEINS

By DAVID I. HITCHCOCK

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1. Hydrogen Ion Concentration in a Pure Ampholyte Solution (page 602). The relation between the value of [H+] at the isoionic point and that prevailing in a solution of a pure amino acid in water is shown by an equation obtained by Walker (92). For a simple ampholyte in pure water, electroneutrality requires that

$$[H^+]+[R^+]=[OH^-]+[R^-]$$

Values for the last three terms may be obtained from equations (1), (2), and (3). Since such a solution will have, in most cases, a low ionic strength, all activity coefficients may be taken as equal to unity. It follows that

$$[\mathrm{H}^+] + \frac{[\mathrm{H}^+]u}{K_1} = \frac{K_w}{[\mathrm{H}^+]} + \frac{K_2u}{[\mathrm{H}^+]}$$

or

$$[H^{+}] = \sqrt{\frac{K_{1}(K_{w} + K_{2}u)}{K_{1} + u}}$$
 (22)

if u denotes the concentration of the electrically neutral ampholyte, $[R^{\pm}]+[R]$. Equation (22) shows that $[H^{+}]$ varies from $\sqrt{K_w}$, its value in pure water, to $\sqrt{K_1K_2}$, its value at the isoionic point, as u is increased from zero to a value much greater than K_1 or K_w/K_2 . Equation (22) may be solved for $[H^{+}]$ by assuming as a first approximation that u is equal to C, the total ampholyte concentration. The approximation may be justified or improved by using this value for $[H^{+}]$ to obtain $[R^{+}]$ and $[R^{-}]$ from equations (2) and (3), and then obtaining a better value for u from the relation,

 $u = C - [R^+] - [R^-]$

Kolthoff (93) has shown that the values so calculated for amino acid solutions agree with those given by the fourth degree equation of Sörensen (16).

- 2. Effect of Salts on the Dissociation of Amino Acids (page 612). The electromotive force of cells without liquid junction, with hydrogen and silver chloride electrodes, was used by Batchelder and Schmidt (94) to study the effects of salts on the apparent or concentration constants for the ionization of amino acids. The amino acids investigated were alanine, aspartic acid, arginine, and ornithine, and the salts were sodium, potassium, lithium, and barium chlorides. The variations in the apparent pK values with ionic strength could be reproduced by equations of the Debye-Hückel type, with empirical terms added (see Chapter XIII, page 1209).
- 3. Electrophoresis of Protein Solutions (page 617). The moving boundary method for the measurement of the migration of a dissolved protein in an electric field was improved by Tiselius (95) (see Chapter XII, page 1191). The new apparatus is used at temperatures near that of the maximum density of water, to avoid the disturbing effects of heat convection. It has an ingenious compensation device to keep the boundaries from passing out of the tube in prolonged experiments, as well as other mechanical and optical improvements. The method has also been described in detail by others (96, 97) who have found it useful. It has been of great value in the characterization of the protein components of biological fluids and in testing the homogeneity of purified proteins.
- 4. Isoelectric Points¹ of Proteins (Table III, page 618). The figures 4.84–4.90 given for egg albumin are the isoionic points found by Sörensen, Linderström-Lang, and Lund (13) for this protein in fairly concentrated solutions of ammonium sulphate and chloride; in the latter case the isoionic point was independent of the salt concentration. Smith (53) found that the isoionic point in sodium acetate buffers depended on the concentrations of salt and protein, but that a double extrapolation to zero concentration gave an isoionic point of 4.86. A similar figure was obtained for the extrapolated isoelectric point of foreign particles coated with albumin by adsorption, and Smith's cataphoresis experiments have been confirmed by Moyer (98), who found pH 4.82 to be the isoelectric

¹ Hill (111) has discussed mathematically the isoelectric point of a mixture of multivalent ampholytes in terms of activity coefficients and thermodynamic equilibrium constants.

point at 0.02 ionic strength. On the other hand Tiselius (54, 99) found that the moving boundary method yielded an isoelectric point at pH 4.55–4.57 for dissolved egg albumin in similar solutions. A comparable low figure was obtained by Adair (100) from measurements of membrane potentials. It has been suggested that the negative charge of the dissolved protein at its isoionic point must be due to its affinity for the anions of the buffer (54), and that the higher isoelectric point of the adsorbed protein may be due to partial denaturation at the surface (98). It seems possible that the latter discrepancy may be connected with the fact discovered by Longsworth (101) that recrystallized egg albumin contains two components.

The isoelectric point of crystallized β -lactoglobulin was located at pH 5.20 by Pedersen (102), who made moving boundary measurements with dilute protein solutions in 0.02 M sodium acetate buffers. This figure is identical with its isoionic point in potassium chloride solutions and with the pH of solutions of the crystals, as reported by Cannan (103).

Recrystallized *pepsin* is now believed not to have an isoelectric point, since Tiselius (104) and Northrop (105) found it to migrate as if always negatively charged, even in 0.1 N hydrochloric acid.

The proteins of blood plasma have been fractionated in various ways, and isoelectric points ranging from pH 5 to 7 have been reported for globulin fractions, according to a review by Cohn (106).

5. Dissociation Curves of Proteins (page 619). The effects of temperature, protein concentration, and salt concentration on the acid-base dissociation curve of egg albumin were investigated by Cannan (103). It was found that the curves were displaced along the pH axis in the direction of neutrality by an increase in salt concentration or in temperature. The point of no apparent displacement was located in the poorly buffered region between pH 6 and 8, and not at the isoionic or isoelectric point. The results were interpreted by an application of electrostatic theory, due to Linderström-Lang, with two empirical corrections.

Electrostatic theory was also used by Kirkwood (107) in a mathematical treatment of acid-base equilibria in solutions of multivalent ampholytes. He concluded that acid-base equilibria and isoelectric points should be sensitive to ionic strength, and that the specific behavior of an ampholyte was to be attributed to electrostatic interaction between its charges and its electrolyte environment.

In studying the combination of acid or base with the insoluble protein of wool fibers, Steinhardt (108) made use of simpler technique and simpler theory. The amounts of bound acid or base were determined by the differences between volumetric titrations, but were plotted against pH for comparison with the electrometric titration curves of soluble proteins. Although the amounts of cation and anion removed by the protein in such experiments were necessarily equivalent, the data could be represented by curves based on the application of the law of mass action to two types of twophase equilibria. One dissociation constant was used to describe the combination between the protein and the hydrogen ion, and another to describe its combination with the anion of the added acid. The specific effects of different ions, supplied either by the acid or base or by an added salt, were thus ascribed to the existence of partially dissociated protein salts rather than to electrostatic forces between ions. Comparative experiments with egg albumin led Steinhardt to suggest that anions (other than OH-) probably combine with dissolved proteins in acid solutions.

The similarity between the dissociation curves of proteins and the curves obtained by plotting electrophoretic mobility against pH has often been pointed out by Abramson (109). He measured the mobility of adsorbed protein by the microscopic observation of foreign particles coated with the protein. These mobilities agreed, in most cases, with those obtained by Tiselius for dissolved proteins; the exceptional behavior of egg albumin has already been mentioned. This protein was investigated over a wide range of pH values by Longsworth (110), who used the Tiselius method. He concluded that, except for minor secondary effects, the mobility of ovalbumin is proportional to the number of equivalents of acid bound by the protein at constant ionic strength at any pH within its stability range, pH 2.6-12.0 at 0°. Such proportionality would be expected only if the friction coefficient of the protein were independent of pH. This was confirmed by the finding that within this pH range the diffusion coefficient of egg albumin, corrected for viscosity, was fairly constant.

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CHAPTER XII

ELECTROCHEMISTRY OF THE AMINO ACIDS AND PROTEINS

By CARL L. A. SCHMIDT

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1. Electrophoresis.¹ The electrophoresis technique for the determination of the mobilities of protein ions introduced by Tiselius (see page 684) has been extended to the separation of proteins. This technique has proven of great importance especially for the estimation of the components of many proteins that at one time

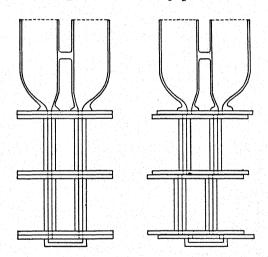


Fig. 1. The assembled U-tube of the new electrophoresis apparatus. (Tiselius, A., The Harvey Lectures, 35, 37 (1939-40).)

were considered as being homogeneous. These components possess properties that are quite similar in many respects. By use of the classical procedures of isolation, they would ordinarily be obtained as apparently homogeneous proteins.

The improvements (69) consist of (a) the use of the schlieren technique for the formation of a sharp boundary initially, (b) the reduction of convective disturbances by the use of a low temperature thermostat so that the experiments may be carried out at

¹ See reference (102).

0°-4°, at which temperature the density of water is maximum or nearly so, and (c) the adaptation of the Foucault-Toepler schlieren effect for observation and photography of the boundaries.

The apparatus consists of an electrophoresis tube built up of glass cells of rectangular cross section (3×25 mm.) with optically plane walls (Fig. 1). These cells can be made to slide over one another thus, as shown in the right side of Fig. 1, separating the contents of the U-tube into five samples. The bottom and the lower of the middle compartments are filled with the protein solu-

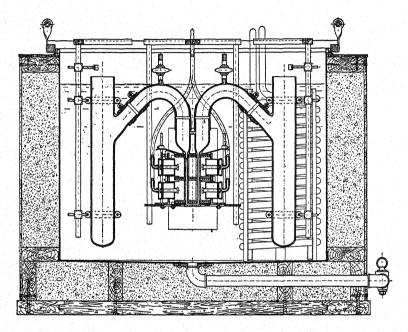


Fig. 2. Complete apparatus in low temperature bath. (Tiselius, A., The Harvey Lectures, 35, 37 (1939-40).)

tion under investigation. The lower of the middle compartments is moved out to one side and the upper part is filled with a buffer solution of the same composition as that of the sample. The protein solution is usually dialyzed against the buffer solution before filling the cells. The lower middle cell is now slid back into alignment with the other parts of the cell thus forming sharp boundaries. The back and forth movements of the cell are carried out mechanically with the aid of air pressure pumps. The U-tube is connected to tubes that contain reversible silver-silver chloride electrodes. The complete assembly except for the silver electrodes is shown in Fig. 2.

On passage of direct current in the appropriate direction through the system, the boundary in one side of the middle section rises and that on the other side descends.

The object of carrying out the electrophoresis at a low temperature (0°-4°) is to minimize the disturbing effects of convection. On passing an electrical current through the system, there will be a difference of temperature between the inside of the tube and the wall of the tube, the temperature of the former being slightly higher. At ordinary temperatures there will be a minimum of density of the solution in the middle of the tube. The heavier solution close to the inside wall of the tube will fall and that in the center will rise, thus setting up a convection current. If the temperature is chosen such that the density variation is a minimum, the effects of convection due to temperature differences can be reduced to a minimum but not wholly eliminated. Since the heavier solution is placed in the lower compartment and the lighter one above, a vertical density gradient is present in the boundary but not in the body of solution on either side of the boundary. Passage of the current tends to establish a horizontal density gradient. If the mean temperature of the solution is near that of maximum density, this may be made quite small. Since no stabilizing vertical gradient exists in the body of the solution, the horizontal gradient must be equalized by circulation of the solution. The extent to which this circulation disturbs the boundary depends upon the relative magnitude of the vertical density gradients in the solutions. Hence it is necessary to use a lower potential for a dilute solution of protein in a given buffer than for a more concentrated one. The boundary disturbances usually appear after prolonged electrophoresis. By an appropriate choice of temperature and of electrical potential, it is possible to minimize somewhat the influence of convection on the sharpness of the boundary.

As a consequence of diffusion there is a progressive blurring of the boundary. Special optical methods are therefore necessary in order to locate the boundary. Tiselius took advantage of the schlieren method whereby small differences of refractive index appear as shadows or schliere. The boundary appears on the screen of the schlieren camera as a dark band. This method does not furnish information as to variation of the refractive index in the boundary. The schlieren scanning method (70) and the cylindrical lens method (71, 72) are now commonly employed. The optical setups are shown in Figs. 3 and 4. The type of buffer employed

depends somewhat on the type of protein under study. Thus, in studying the proteins of animal sera, a 0.2μ phosphate buffer of pH 7.7 and a buffer containing 0.025 N each of lithium diethylbarbiturate, diethylbarbituric acid, and lithium chloride of pH 7.85 have both been employed.

Examples of the type of protein differentiation that are obtained in sera and plasmas are shown in Fig. 5. By means of the electro phoresis technique it has been possible to fractionate a good many

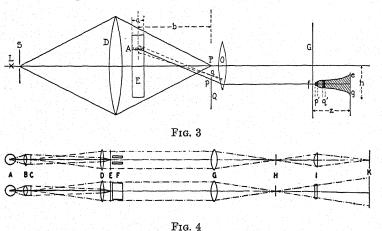


Fig. 3. Diagram of Longsworth's "schlieren scanning" method. L, lamp; S, horizontal slit; D, schlieren lens; E, electrophoresis cell; Q, schlieren diaphragm, the movement of which is mechanically coupled to the movement of the plate, G, at

right angle; O, camera lens.

Fig. 4. Diagram of the Philpot-Svensson method for observation of concentration gradients. Upper half of diagram shows arrangement viewed from above, lower half from the side. A, lamp; B, condensing lens; C, slit; D, schlieren lens; E, screen; F, electrophoresis cell; G, camera lens; H, tilted slit; I, cylindric lens; K, photographic plate or screen.

(Tiselius, A., The Harvey Lectures, 35, 37 (1939-40).)

proteins that are closely related as well as mixtures of proteins whose properties are widely separated. A few examples of proteins that have been studied electrophoretically are: hemocyanin of several types of snails (73); egg white (74, 75); horse serum (76); yellow enzyme (77); rat and human sera (78); pepsin (79); animal viruses (80); tetanus antitoxin (81); and many others (82).

The electrophoresis method is also employed for the preparation of proteins (83). There probably are limitations to the use of electrophoresis for differentiating and isolating proteins. Thus it is not possible to differentiate a particular type of protein obtained from certain closely related biological species as well by this method as

by serological reactions or solubilities of the crystalline material (101). This may be due to the fact that species differences are due to differences in groups that are electrically inert.

2. Mobilities of Proteins. As has already been pointed out on page 684, the electrophoresis technique lends itself to the determination of mobilities of proteins as functions of pH and of the ionic strength of the buffer solutions in which they are dissolved (Fig. 6). At constant ionic strength, mobility values are plotted against values of pH. When such a curve for egg albumin is compared with

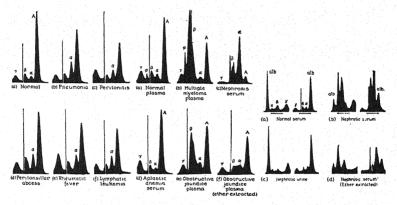


Fig. 5. Electrophoresis diagram of normal and pathological sera and plasmas. (Longsworth, L. G., Shedlovsky, T., and MacInnes, D. A., J. Exp. Med., 70, 399 (1939).)

the titration curve carried out at the same ionic strength (0.1) (84), it is seen that a constant proportionality exists between the mobility and the charge on the protein over the entire pH-stability range. The numerical value of the proportionality constant differs by 40 per cent from the value computed by the Debye-Hückel-Henry theory. The discrepancy may be due to the fact that the ovalbumin molecules are not spherical and hence the theory which was developed for spherical particles, applies only in a general way.¹ The reduction in mobility caused by increasing ionic strengths, can be accounted for by the Debye-Hückel-Henry theory (85).² The displacement of the isoelectric point by variation of the

¹ In this connection, see Bull, H. B., Trans. Faraday Soc., 36, 80 (1940).

² According to Gorin (Gorin, M. H., J. Phys. Chem., 45, 371 (1941)) the apparent agreement with the simple equation $v = Qf(\kappa r)/6\pi\eta r(1+\kappa r)$, where Q = net charge per ion, v = electrical mobility, $f(\kappa r)$ is the Henry function, $\kappa = \text{the Debye-Hückel ionic strength function}$, and η is the viscosity of the medium, found by Tiselius and Svensson (85) is, to a certain extent, fortuitous. By assuming a non-spherical

ionic strength may be partially explained by the combination of ions other than H⁺ and OH⁻ with the protein. Increase of protein concentration and decreasing ionic strength may influence the amount of protein-protein complexes that are present in the solu-

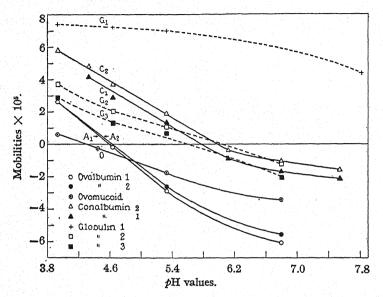


Fig. 6. The electrophoretic mobilities of the constituents of egg white as functions of the pH.

(Longsworth, L. G., Cannan, R. K., and MacInnes, D. A., J. Amer. Chem. Soc., 62, 2580 (1940).)

tion especially when the components carry opposite electrical charges (75, 86).3

3. Diffusion Constants of Proteins. 4 The method of determining

molecule rather than a sphere, it is possible to explain the results of Tiselius and Svensson from $\mu = 0.04$ M to $\mu = 0.20$ M within 4 per cent. Gorin has shown that if the egg albumin molecule is a sphere, the results predicted for a sphere, when the corrections due to the finite size of the other ions is considered, are markedly different from those given by the above equation at high values of the ionic strength. At low values of the ionic strength, deviations occur. In this connection, see also Abramson, H. A., Gorin, M. H., and Moyer, L. S., Chem. Rev., 24, 345 (1939).

³ Adair, G. S., and Adair, M. E., *Trans. Faraday Soc.*, 36, 23 (1940) have shown that calculation of the mobilities of the ions of hemoglobin from the measurements of the charge and radius, Henry's formula being used, are in fairly close agreement with the observed mobilities.

⁴ A more recent review of diffusion measurements of proteins is given by Neurath, H., Chem. Rev., 30, 357 (1942). See also Longsworth, L. G., Ann. N. Y. Acad. Sci., 41, 267 (1941).

diffusion constants developed by Lamm and Polson (87) has come into general use. The diffusion cell is shown in Fig. 7. The protein solution which has previously been dialyzed against an appropriate buffer solution is placed in limb d and the buffer solution or solvent in limb a. Capillary tube b is inserted in limb d. It serves to regulate the rate of flow of the solution contained in d to limb a.

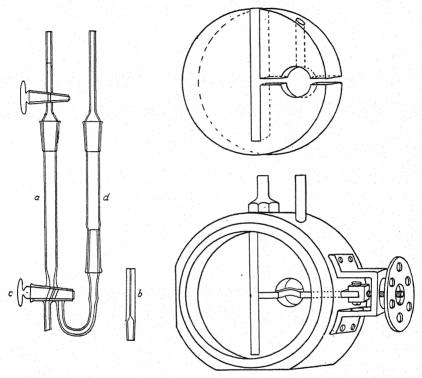


Fig. 7. Types of diffusion cells.¹ (Polson, A., *Kolloid Z.*, **87**, 149 (1939).)

¹ An improved cell for optical diffusion measurements on solutions has been described by Neurath, H., *Science*, 93, 431 (1941). See also Neurath, H., *Chem. Rev.*, 30, 357 (1942).

The cell is placed in a water thermostat and, after temperature equilibrium has been attained, stopcock c is opened. Air pressure is applied to d and the protein solution is forced through capillary b until a suitable boundary in limb a is obtained. The stopcock is then closed. The process of diffusion is measured refractometrically by photographing a uniform transparent scale through the diffusion cell. The refractive index gradient at the diffusion boundary produces a distorted image of the scale in which the scale line dis-

placement is proportional to the concentration gradient when the refractive index is a linear function of the concentration. An ultraviolet lamp is used as a source of light. The light just passes through a filter that transmits light of a wavelength not absorbed by the solution under examination. Infra-red light is also, at times, employed. A second type of cell is described by Polson (88) (see Fig. 7).

On completion of the experiment, the rate of change of refractive

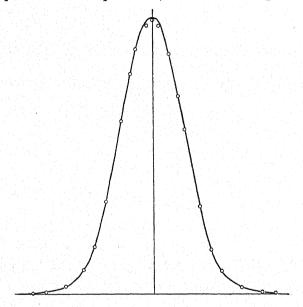


Fig. 8. Diffusion gradient of human hemoglobin. The circles indicate ideal values.

(Polson, A., Kolloid Z., 87, 149 (1939).)

index with height is plotted against the height. A curve of the type shown in Fig. 8 is obtained. The equation for this type of curve was developed by Wiener (89):

$$\frac{dn}{dx} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \cdot e^{-x^2/4Dt}$$

where n_1 is the refractive index of the solution, n_0 is the refractive index of the solvent, D is the diffusion constant, t is the time since the diffusion started, and x is the distance of a point in the cell from the original boundary. A number of equations based on the above have been derived that permit calculating the diffusion constant under the particular conditions of the experiment (87,

TABLE I

Protein	v	$S \cdot 10^{13}$ cm./sec.	$D \cdot 10^7$ cm. 2 /sec.	M_s	$M_{\mathfrak{g}}$
Cytochromeic	0.71	1.89	10.11	15600	
Lampetra Erythrocruorin	0.751	1.90	10.65	17400	19000
Myoglobin	0.745	2.04	11.25	17200	
Lactalbumin	(0.751)	1.90	10.57	17500	
Gliadin	0.722	2.10	6.72	27400	
Pepsin	0.750	3.30	9.00	35500	39200
Insulin	0.749	3.47	8.20	40900	
Insulin (oxid.)	0.749	3.47	7.13	43300	
Bence-Jones protein	0.750	2.85	7.33	37700	
Lactglobulin	0.751	3.12	7.27	41800	37800
Ovalbumin	0.749	3.55	7.76	43800	40500
CO-hemoglobin	0.749	4.50	6.90	63000	68000
Serum albumin	0.748	4.50	6.17	70100	67100
Yellow enzyme	0.731	5.76	6.28	82800	77500
Serum globulin	0.745	7.10	4.05	167000	150000
Phycocyan, pH 5.4	0.750	11.50	4.15	269000	
Phycocyan, pH 7.5	0.750	6.20	4.58	131000	
Phycoerythrin	0.750	12.0	4.06	286000	292000
Edestin	0.744	12.8	3.93	309000	
Excelsin	0.743	13.3	4.26	294000	
Amandin	0.746	12.5	3.62	329000	329000
Palinurus hemocyanin	0.740	16.4	3.39	446000	460000
Thyroglobulin (pig)	0.720	19.2	2.65	628000	675000
Homarus hemocyanin	0.740	22.6	2.78	752000	784000
Nephrops hemocyanin	0.740	24.5	2.79	819000	766000
Planorbis erythrocruorin	0.745	33.7	1.96	1630000	1539000
Eledone hemocyanin	0.740	49.1	1.64	2790000	
Eledone hemocyanin, pH 11.6	0.740	10.6	2.25	440000	
Octopus hemocyanin	0.740	49.3	1.65	2785000	
Lumbreius erythrocruorin	0.740	60.9	1.81	3140000	2946000
Rossia hemocyanin	0.740	56.2	1.58	3316000	
Helix hemocyanin	0.738	98.9	1.38	6630000	6706000
Helix hemocyanin, pH 8.6	0.738	16.0	1.82	813000	

V= specific volume; S= sedimentation constant; D= diffusion constant at infinite dilution; $M_s=$ Molecular weight calculated from specific volume, diffusion constant, and sedimentation constant data; $M_g=$ molecular weight from sedimentation equilibrium data obtained from ultracentrifuge. (Polson, A., Kolloid Z., 87, 149 (1939).)

88). The diffusion constants may also be calculated by a statistical method that is used for obtaining dispersion. The assumption is made that the curves obtained from diffusion may be treated as ideal dispersions (87, 90).

Table I summarizes data collected by Polson (88) for a number of proteins. Proteins of large molecular weight (1,000,000) show slight

TABLE II. Activity Coefficients of Amino

m	dl- Alanine	dl-α- Amino-n- butyric acid	dl-α- Amino-n- valeric acid	α-Amino- iso- butyric acid	dl-Valine	β-Alanine	dl-β- Amino butyric acid	dl-β- Amino valeric acid	γ-Amino- butyric acid	dl-γ- Amino- valerie acid	ε-Amino- caproic acid
0.1											
0.2	1.005	1.020	1.022	1.025	1.030	0.994	1.006	1.016	0.983	1.001	0.971
0.3	1.008	1.029	1.032	1.038	1.045						
0.4	1.010	1.039	1.044	1.050	1.060						
0.5	1.012	1.048	1.055	1.062	1.076	0.988	1.019	1.044	0.966	1.009	0.951
0.65	1.015	1.062	1.072	1.080	1.101						
0.7						0.987	1.029	1.064	0.961	1.018	0.946
0.8	1.018	1.077		1.099						100	
1.0	1.023	1.096		1.121		0.988	1.049	1.095	0.960	1.037	0.942
1.2	1.027	1.114		1.144		0.991	1.064	1.118	0.964		0.964
1.5	1.034	1.141		1.177		1.000	1.089	1.154	0.975	1.079	1.002
1.7	1.040	1.159					1 1 A	1.5		100	
1.9	1.046	1.178				1000				100	
2.0				Bulletin in		1.016	1.138	1.218	1.006	1.133	1.072
2.1		1.195					7 - 12 - 1				
2.3											
2.5					100	1.042	1.193	1.289	1.050	1.199	1.140
3.0		100				1.073	1.254	1.364	1.104	1.273	1.208
3.5						1.109	1.319	1.443	1.165	1.355	1.271
4.0	Last to the					1.149	1.388	1.523	1.230	1.440	1.330
5.0						1.239	1.525	1.681	1.374	1.622	1.436
6.0						1.339	1.662	1.818	1.506	1.812	1.517
6.5								1.870	1.575		1.556
7.0	1.0			1000		1.445	1.790			2.014	
7.3							100				1

(Smith P. K., and Smith, E. R. B., J. Biol. Chem., 117, 209 (1937); 121, 607 (1937); 132, 47 and 57 (1940); 135, 273 (1940).)

changes in the diffusion constant when the concentration is changed. The smaller the molecular weight, the greater is the change.

Measurement of the diffusion constants of hemoglobin and of pepsin was one of the criteria employed by Steinhardt (90) in order to determine whether or not these proteins are dissociated into smaller molecules in the presence of amides. Neurath and Saum (92) also used diffusion as well as viscosity measurements to determine the effect of urea and of heat denaturation on serum albumin. The diffusion constant was found to decrease with increasing concentration of urea. Heat denaturation in the presence of urea or at pH 3.2 likewise leads to a decrease in diffusion constant. On the assumption that there is no change in molecular weight under these conditions, it is calculated that protein molecules become more elongated with increase in urea concentration. The diffusion constant of bushy stunt virus, which appears to have a molecular weight of 10,600,000 at 20°, has a value 1.15×10⁻⁷ (93).

4. Activity Coefficients. Further work on the determination of

Acids and Related Compounds

dl-Serine	dl-Thre- onine	dl-Pro- line	l-Hy- droxy- proline	Sarco- sine	Betaine	Glycyl- glycine	Glycyl- alanine	Alanyl- glycine	Alanyl- alanine	Tri- glycine
										0.910
0.964	0.988	1.019	1.001	1.004	1.071	0.912	0.935	0.929	0.981	0.852
0.945	0.983	1.028	1.001	1.006	1.108	0.878	0.912	0.906	0.979	0.804
0.907	0.974	1.047	1.002	1.012	1.186	0.827	0.882	0.878	0.985	
	0.967	1.068	1.003	1.020	1.269	0.791	0.868	0.865	1.002	
	0.959	1.096	1.007	1.032	1.403	0.744	0.854	0.855	1.036	
	0.955		1.010	1.042	1.499	0.723	0.845	1.75		
	0.950	1.148	1.015	1.059	1.654	0.696				
				8 1 1		0.685			1 to 100	
	0.943	1.206	1.026	1.091	1.945					
			1.034							
		1.269		1.128	2.282	1		100		
		1.338		1.173	2.672					
		1.406		1.221	3.091					
		1.495	1	1.275	3.643					
		1.672		1.391	3.933					1 - 2
		1.828		1.513						
		1.979		1.627						
		2.002								Large et al.
				100					A seed to	

the activity and osmotic coefficients of amino acids by the isopiestic method has been carried out by Smith and Smith (94). Their values are given in Tables II and III. In general the values for the activity and osmotic coefficients increase with each additional methylene group although not invariably so. There is a considerable difference in the osmotic coefficients of amino acids of the same empirical formula. These differences appear to be related to differences in dipole distance. In general the larger the dipole moment, the lower will be the osmotic coefficient. The effect is opposite to that due to increasing the length of the carbon chain. When strongly polar groups are contained in the hydrocarbon chain, the values for the osmotic coefficients are considerably depressed. The osmotic coefficients of amino acids in which methyl groups are attached to the nitrogen are much higher than are their straight chain analogues.

Certain functions derived from change of activity coefficient with concentration and solvent are given in Tables IV and V. The data indicate that the values for $-\log \gamma/C$ at $D_0/D=1$ decrease with an increase in length of the carbon chain and increase with an increase

in dipole moment. The effect of substitution of an hydroxyl group is to decrease the values for this quantity while substitution of a methyl group to the nitrogen leads to an increase. In the case of peptides that contain the same amino acid, the value for the function increases with each added amino acid residue. The values for the peptides that contain different amino acids are much larger than that of either component amino acid. The function is influenced by

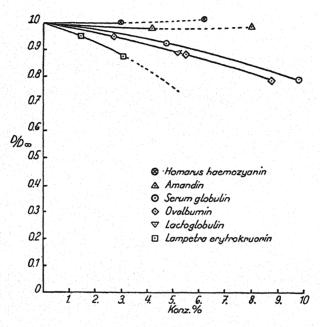


Fig. 9. Effect of concentration on D/D_∞. (Polson, A., Kolloid Z., 87, 149 (1939).)

the nature of the amino acids in the peptide, their number, and the arrangement. In general, the values for N_A/N_0 parallel those of the previous function indicating the influence of the factors just mentioned.

From the equation $-\log \gamma/C = K_R^*(D_0/D) - K_s^*$ in which K_R^* , the salting-in constant, is the slope of the line and K_s^* , the salting-out constant, is the intercept, it is possible to calculate values for K_R^* and K_s^* when the values for $-\log \gamma/C$ vary linearly with D_0/D (95). If the curves are not straight lines, it is necessary to use the slopes obtained from measurements in dilute solutions. The values for K_R^* and K_s^* in Tables IV and V were so obtained. In general, the values for the salting-in constants increase with in-

Table III

Equations for Osmotic Coefficients of Amino Acids and Related Compounds

Sucrose $ \phi = 1 + 0.084m + 0.0104m^2 - 0.00237 $ Glycine $ dl\text{-Alanine} $ $ dl\text{-Alanine} $ $ dl-\alpha\text{-Amino-}n\text{-butyric acid} $ $ dl-\alpha\text{-Amino-}n\text{-valeric acid} $	
dl-Alanine $ \begin{aligned} \phi &= 1 + 0.010m - 0.001m^2 \\ dl-\alpha-\text{Amino-}n\text{-butyric acid} \\ dl-\alpha-\text{Amino-}n\text{-valeric acid} \end{aligned} $	
dl - α -Amino- n -butyric acid $\phi = 1 + 0.049m - 0.004m^2$ dl - α -Amino- n -valeric acid $\phi = 1 + 0.054m$	
dl - α -Amino- n -valeric acid $\phi = 1 + 0.054m$	
A	
α -Aminoisobutyric acid $\phi = 1 + 0.067m - 0.010m^2$	
dl -Valine $\phi = 1 + 0.073m$	
β -Alanine $\phi = 1 - 0.018985m + 0.019307m^2 - 0.$	$002431m^3$
$+0.000109m^4$	
dl - β -Amino- n -butyric acid $\phi = 1 + 0.008244m + 0.02395m^2 - 0.0$	$0503m^3$
$+0.0003335m^4$	
dl - β -Amino- n -valeric acid $\phi = 1 + 0.0579m - 0.002374m^2 + 0.00$	$0775m^3$
$-0.000102m^4$	
γ -Aminobutyric acid $\phi = 1 - 0.0402m + 0.04616m^2 - 0.0075$	
$dl-\gamma$ -Amino-n-valeric acid $\phi = 1 - 0.000675m + 0.02885m^2 - 0.0$	$0047m^3$
$+0.000246m^4$	
$\phi = 1 + 0.04635m - 0.002009m^2 + 0.0000000000000000000000000000000000$	$001646m^3$
$-0.0001904m^4$	
<i>l</i> -Hydroxyproline $\phi = 1 + 0.0007326m + 0.0044m^2 - 0.0044m^2$	
dl-Serine $\phi = 1 - 0.0854m - 0.0505m^2 + 0.0425$	
dl-Threonine $\phi = 1 - 0.0323m + 0.0185m^2 - 0.0038$	
Sarcosine $\phi = 1 + 0.008847m + 0.009898m^2 - 0.$	$0007366m^3$
$-0.000009m^4$	
Betaine $\phi = 1 + 0.17277m - 0.05794m^2 + 0.00$	$005224m^{3}$
$-0.0000341m^4$	
Glycylglycine $\phi = 1 - 0.2628m + 0.2359m^2 - 0.1142$	
Alanylglycine $\phi = 1 - 0.2321m + 0.3398m^2 - 0.1517$	
Glycylalanine $\phi = 1 - 0.2047m + 0.2569m^2 - 0.1009$	
Alanylalanine $\phi = 1 - 0.0765m + 0.2038m^2 - 0.0883$	$3m^3$
Triglycine $\phi = 1 - 0.572m + 1.555m^2 - 2.35m^3$	
Potassium chloride $\phi = 1 + 0.084m + 0.0104m^2 - 0.00237$	$7m^3+0.000115m^4$

Note: By use of empirical equations for the osmotic coefficients, $\phi=1+am+bm^2+cm^3+dm^4$, where a may be positive or negative, and b, c, and d may be positive, negative, or 0, the activity coefficients may be calculated from the equation, 2.3026 $\log \gamma=2$ $am+3/2bm^2+4/3cm^3+5/4cm^4$.

(Smith, P. K., and Smith, E. R. B., J. Biol. Chem., 121, 607 (1937); 132, 47 and 57 (1940); 135, 273 (1940).)

crease in dipole moment and decrease with increase in length of the carbon chain while the reverse holds for the salting-out constants. The substitution of an hydroxyl group in alanine, as in serine, leads to a decrease in the value for K_R^* , while in the case of hydroxyproline and threonine the substitution of an hydroxyl group does not change this quantity over their respective unsubstituted analogues. The substitution of an hydroxyl group, as in

Table IV

Functions Derived from Change of Activity Coefficient with Concentration and Solvent

Compound	$\frac{-\operatorname{Log}\gamma}{C} \text{ at}$ $\frac{D_0}{D} = 1$	$-\log \frac{N_A}{N_0}$	K_R*	K_z*
dl-α-Alanine	-0.005	2.8561	0.010	0.015
Sarcosine	-0.008		0.016	0.024
dl-Serine	0.075	3.3622	-0.102	-0.177
dl-α-Amino-n-butyric acid	-0.033	2.375^{1}	0.016	0.049
dl-Threonine	0.027	3.0702	0.014	-0.013
dl-α-Amino-n-valeric acid	-0.042	$(2.158)^3$	0.000	0.042
Betaine	-1.150	0.075^{2}	0.042	0.192
dl-Proline	-0.043	$(1.50)^4$	0.004	0.047
l-Hydroxyproline	-0.001	$(2.3)^5$	0.008	0.009
Glycine	0.096	3.391	0.092	-0.004
Glycylglycine	0.241	4.367	0.227	-0.014
Tryglycine	0.470	7.206	0.487	0.017
Alanine	-0.005	2.856	0.010	0.015
Alanylalanine	0.073		0.203	0.130
Alanylglycine	0.208		0.293	0.085
Glycylalanine	0.189		0.243	0.054
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¹ Cohn, E. J., McMeekin, T. L., Edsall, J. T., and Weare, J. H., *J. Amer. Chem. Soc.*, **56**, 2270 (1934).

 $C = \text{concentration of amino acid; } D_0 = \text{dielectric constant of solution; } D = \text{dielectric constant of the solvent; } K_R^* = \text{salting in constant; } K_s^* = \text{salting out constant; } N_A = \text{solubility in mole fractions in alcohol; } N_0 = \text{solubility in mole fractions in water; } \gamma = \text{activity coefficient.}$

(Smith, P. K., and Smith, E. R. B., J. Biol. Chem., 132, 57 (1940) and 135, 273 (1940).)

these three amino acids, decreases the values for K_s^* . The values for K_R^* increase with the number of peptide bonds contained in the peptide. K_s^* shows a less regular relation.

² Edsall, J. T., in Schmidt, C. L. A., The Chemistry of the Amino Acids and Proteins, Springfield and Baltimore, 1938, pages 904, 906, and 908.

³ This is the ratio for volume; that for α -amino-n-valeric acid is probably about 2.00.

⁴ From the solubility equation of Tomiyama. T., and Schmidt, C. L. A., *J. Gen. Physiol.*, 19, 379 (1935) for aqueous solutions of *l*-proline at 19° and the measurement of Kapfhammer, J., and Eck, R., *Z. physiol. Chem.*, 170, 294 (1927) in alcohol at 19°.

⁵ From the solubility equation for aqueous solutions at 40° (Wyman, Jr., J., and McMeekin, T. L., J. Amer. Chem. Soc., 55, 908 (1933)) and the solubility of *l*-hydroxyproline in a 0.005 M alcoholic solution of *l*-proline at 40° (Kapfhammer, J., and Eck, R., Z. physiol. Chem., 170, 294 (1927)).

Table V
Functions Derived from Change of Activity Coefficient with Concentration and Solvent

NCH_2		$rac{{ m Log}\; \gamma}{C}$ at	$\frac{D_0}{D} = 1$			$-\mathrm{Log}$	$\frac{N_{A^1}}{N_0}$	
	α	β	γ	€	α	β	γ	é
0	0.096				3.391			
1	-0.005	0.015			2.856	3.139		
2 3	-0.033	-0.011	0.043		2.375			
3	-0.041	-0.035	-0.011	14 24				100
4				0.079	1.414			2.972
		K_R*				K_s	*	
0	0.092				-0.004			
1	0.010	0.022			0.015	0.007		
2	0.016	0.024	0.046		0.049	0.035	0.003	
3	0.000	0.012	0.018		0.042	0.047	0.019	
4				0.098				0.019

The Greek letters at the head of the columns denote the positions of the amino group in various amino acids.

(Smith, E. R. B., and Smith, P. K., J. Biol. Chem., 132, 47 (1940).)

- 5. Electrodes. The glass electrode has come to be generally used in amino acid and protein research. A complete description is given by Dole (96). A recently described direct reading pH meter (97) is used in the writer's laboratory in conjunction with the glass electrode. Various attempts to determine the activity of calcium ions in the presence of proteins have not been successful. Calcite and fluorite crystals as calcium electrodes, due to high resistance, are not adapted (98). The antimony electrode can be used for the estimation of pH when proteins are present in the solution (99).
- 6. Transference. Shedlovsky (100) has proposed the use of the equation, $1/t = 1/t_0 + AC^{\frac{1}{2}} BC$, for the calculation of transference numbers. It has not yet been applied to amino acids and proteins.

 $^{^{1}}$ N_{A} = solubility in mole fractions in alcohol. N_{0} = solubility in mole fractions in water.

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CHAPTER XIII

COMBINATION OF AMINO ACIDS AND PROTEINS WITH ACIDS, BASES, HEAVY METALS, AND OTHER COMPOUNDS

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1. Combination with Acids and Bases. It is now definitely established that the combination of proteins with acids and bases, respectively, is due to the presence of specific free groups in the protein molecule. In support of this statement, it was necessary that stoichiometric relations be established. Cannan, Kibrick, and Palmer (70) made a study of egg albumin with this regard. On the basis of an analysis of dissociation curves, it was estimated that the numbers of the various ionizable groups in egg albumin are: (a) total cations, 41; (b) carboxyl minus guanidino, 37; (c) amino, 22; (d) imidazole, 5; (e) guanidino (a-c-d), 14; (f) carboxyl (b+e), 51. Egg albumin probably contains one mole of phosphoric acid. This group will contribute two equivalents to the curve. One of these is submerged in the carboxyl region close to pH 2 and the other in the imidazole region at about pH 7. The estimates of carboxyl and imidazole groups include the phosphoric acid. The ionizable groups may also be estimated on the basis of the content of certain amino acids. The guanidino group of arginine will contribute 14 equivalents per mole, the imidazole group of histidine 4, the ε-amino group of lysine 15, and the hydroxyl group of tyrosine 10. The free carboxyl groups are calculated on the basis that glutamic acid contributes 40 and the aspartic acid 24, making a total of 64. However, 31 of these groups are present as acid amides. This leaves 33 free carboxyl groups. The values for the number of basic groups calculated from the dissociation curves do not wholly check with those calculated on the basis of the analytical data. The agreement is, however, approximate. It is possible that diamino acids other than lysine are present in egg albumin and, if so, the discrepancy is apparent rather than real. There is a large discrepancy between the free carboxyl groups as shown by the dissociation curves and those calculated from the amounts of aspartic and glutamic acids determined analytically. Since the curves were not subjected to analyses above pH 9, it is improbable that the phenolic hydroxyl group contributes to the dissociation curve. The discrepancy may, in part, be due to analytical errors in the estimation of the dicarboxylic amino acids. It is also possible that hydroxydicarboxylic amino acids may be present in this protein. The discrepancies are not to be taken as indicating the non-validity of the ideas relating to the seats of acid and base combination but rather that our knowledge of the amino acid content of proteins is still incomplete.

Wool offers certain advantages for the study of acid and base combining capacity. Since wool protein is insoluble, the amounts of acid and base with which it combines can be determined in the solutions in which it is immersed. It is not necessary to make assumptions, when calculating the amounts bound, as to relations between activity and concentration. Wool protein has almost the same equivalent numbers of basic and acidic groups. It contains only a very small amount of histidine. Finally, its state is not affected by exposure to acid in the pH range that is usually investigated. Steinhardt and coworkers (71) have studied the combination of wool proteins with a large number of acids. The sum of the acid-binding and base-binding capacities of wool between pH 0.8 and pH 14.3 is 1.59±0.04 millimoles per gram. On the basis of the content of the free carboxyl groups of aspartic and glutamic acids and the free basic groups of arginine, lysine, and histidine, as determined analytically, the expected value is 1.46. With the hydroxyl group of tyrosine included it is 1.78. It is probable that at least all of the tyrosine hydroxyl groups are not free to combine with base. If this is so, then the discrepancy between 1.59 and 1.46 indicates that some hydroxyl groups are free. If they are not free, then the likelihood is that certain of the analytical values are low. It appears improbable, over the pH range studied, that the SHgroup of cysteine would be appreciably titrated. If the protein contains phosphates and it is treated with a divalent cation, partially dissociated phosphates will be formed and this must be taken into consideration in studying base-combining capacities of proteins. The anions of the carboxylic amino acids may act similarly (72).

2. Salt Effect. The presence of a neutral salt will affect the titration curve of amino acids and proteins and hence the value of the ionization constant. Batchelder and Schmidt (73) carried out ex-

periments to determine the effect of neutral salts on the dissociation of amino acids. Cells with and without a liquid junction were used. The reason for not employing a cell with a liquid junction is that the added salt will influence the value of the liquid-junction potential. Formulas for the calculation of liquid-junction potentials are accurate only for solutions at high dilutions or under certain specialized conditions. At higher salt concentrations, the liquid-junction potential cannot be calculated without extrathermodynamic assumptions. Measurements of the electromotive forces of any cells that involve salt bridges must therefore be regarded as of limited quantitative value. Cells of the type

 $H_2(Pt)$ amino acid (m_T) , NaOH or HCl $(m_B \text{ or } m_A)$, MCl (m_s) Ag Cl Ag

are free from these objections. The concentrations m_B , m_A , and m_T of the base, acid, and amino acid, respectively, can be maintained constant, while the concentration m_s of the salt MCl, can be varied over a range of ionic strength.

The effects of salts on the ionization of amino acids can be explained in the same manner as the effects of salts on the ionization or activity coefficients of other electrolytes. On the assumption that none but electrostatic forces determine the effects of salts on the ionization of proteins as well as of amino acids, the following method may be employed to obtain a limiting equation relating the pH of an ampholyte solution to the salt concentration. Each of the steps in the ionization of amino acids and proteins can be represented by the following general equation:

$$AH_n^+ \rightleftharpoons AH_m^+ + H^+ \tag{1}$$

where A represents the isoelectric ampholyte. The value of n is greater than that of m by unity. The thermodynamic equilibrium constant is given by

$$K = \frac{\alpha_{AH_{m}^{+}}\alpha_{H^{+}}}{\alpha_{AH_{m}^{+}}} = \frac{c_{AH_{m}^{+}}c_{H^{+}}}{c_{AH_{m}^{+}}} \cdot \frac{\gamma_{AH_{m}^{+}}\gamma_{H^{+}}}{\gamma_{AH_{m}^{+}}}$$
(2)

Under the restriction that the value of the ratio $c_{AH_m^+}/c_{AH_n^+}$ is constant, and incorporating its value with that of K,

$$-\log c_{\rm H} + \gamma_{\rm H} = -\log \alpha_{\rm H} = -\log K - \log \gamma_{\rm AH} + \log \gamma_{\rm AH}$$
(3)

Since

$$-\log \gamma_{\rm AH_n^+} = 0.5n^2 \sqrt{\mu}; -\log \gamma_{\rm AH_m^+} = 0.5m^2 \sqrt{\mu}; -\log \gamma_{\rm H^+} = 0.5\sqrt{\mu}$$
 (4)

$$-\log \alpha_{\rm H}^{+} = -\log K + (n^2 - m^2)0.5\sqrt{\mu}$$
 (5)

Or, since n-m=1

$$-\log \alpha_{\rm H} = -\log K + (m+n)0.5\sqrt{\mu}$$
 (6)

$$-\log c_{H^{+}} = -\log K + (m+n-1)0.5\sqrt{\mu}$$
 (7)

Equations (6) and (7) are limiting equations. In a more detailed treatment it would be necessary to account for the finite size of the ions, the spatial location of the electrical charges on the ions, and any specific effects of the salt.

Fig. 1 illustrates the effects of salts on the values for $-\log K_1$ and $-\log K_2$ of alanine. Similar relationships are shown in Fig. 2 for ornithine and in Fig. 3 for aspartic acid. At low salt concentrations, the form of the curves showing the changes in the values of

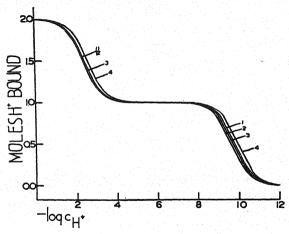


Fig. 1. The dissociation curves of alanine in solutions of sodium chloride. Curve 1 = infinite dilution; curve 2 = 0.5 M sodium chloride; curve 3 = 1.0 M sodium chloride; curve 4 = 1.5 M sodium chloride.

(Batchelder, A. C., and Schmidt, C. L. A., J. Phys. Chem., 44, 893 (1940).)

the apparent dissociation constants can be predicted on the basis of the theory of interionic attraction. Since the activity coefficient of the electrically neutral portion of the amino acid and the "salting out" term are proportional to the first power of the ionic strength, the chief factors that determine the shape of the curves at low salt concentrations are the activity coefficients of the other ionic species that are involved in the ionization equilibrium. At higher values of the ionic strength, the activity coefficient of the zwitterionic portion of the amino acid plays a relatively more important part in determining the course of the theoretical curve.

When the above equations are applied to the ionization of pro-

teins, m+n-1 will approximately equal m+n, except possibly in certain limited pH regions immediately on either side of the isoelectric point. The limiting slope of the $-\log c_H^+$: $\sqrt{\mu}$ curve will

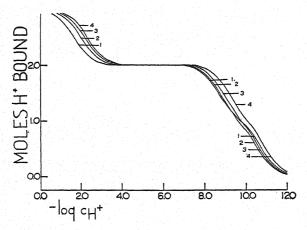


Fig. 2. The dissociation curves of ornithine in solutions of sodium chloride Curve 1 = infinite dilution; curve 2 = 0.5 M sodium chloride; curve 3 = 1.0 M sodium chloride; curve 4 = 1.5 M sodium chloride.

(Batchelder, A. C., and Schmidt, C. L. A., J. Phys. Chem., 54, 893 (1940).)

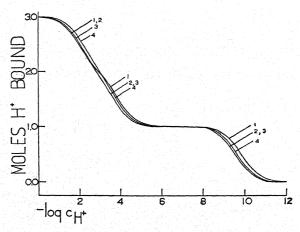


Fig. 3. The dissociation of aspartic acid in solutions of sodium chloride. Curve $1=\inf$ infinite dilution; curve 2=0.5 M sodium chloride; curve 3=1.0 M sodium chloride; curve 4=1.5 M sodium chloride.

(Batchelder, A. C., and Schmidt, C. L. A., J. Phys. Chem., 44, 893 (1940).)

differ only slightly from the limiting slope of the $-\log \alpha_{H}^+$: $\sqrt{\mu}$ curve, provided the pH of the solution does not approximate too closely that of the isoelectric point of the protein. According to

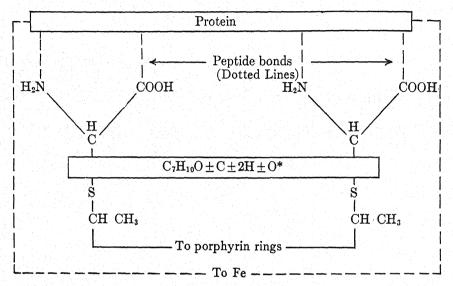
equation (6), addition of salts to a protein solution will cause an increase in the pH of the solution if the initial pH was less than that of the isoelectric point and a decrease in pH if the initial pH was greater than that of the isoelectric point. Results in accord with this have been observed on various occasions (74). In more recent times studies on the salt effects on the dissociation of proteins have been carried out by Cannan and coworkers (70) and by Steinhardt and coworkers (75).

3. Combination with Heavy Metals. It was previously pointed out (page 746) that all heavy metals form complexes with proteins and hence the activity of the heavy metal is very small. In fact all metallic ions except possibly the alkali elements form complex ions with proteins (76). It has even been indicated that the elements of this group may also form partially ionized protein compounds (77). In recent times certain of the radioactive elements have been employed in order to determine the amount of metallic element that is combined with a protein. Thus Cohn and coworkers (78) crystallized insulin from solutions containing radioactive zinc. The amount of zinc in the crystals separated from acetate buffer at pH 5.9-6.0 was independent of the amount of zinc in the solution in which the crystals were formed. The crystals separated from the precipitates in systems of low ionic strength to pH 5.5 contained about 0.34 per cent of radioactive zinc. When the reaction was less acid and up to pH 6.4, the amount of zinc was larger. Two atoms of zinc combine with each molecule of insulin. The molecular weight of insulin is about 37,300.

A number of oxidation enzymes contain a metallic element in the prosthetic group (79). The prosthetic group is a substance of comparatively low molecular weight combined with a protein. Different members of a particular group of enzymes contain the same or a very similar prosthetic group but differ from each other in respect to the protein moiety and the reaction which it catalyzes. The protein moiety determines the nature of the catalysis. Some of the protein compounds that contain iron in the prosthetic group are: hemoglobin (Fe-protoporphyrin); peroxidase (Fe-protoporphyrin); cytochrome c (slightly modified Fe-protoporphyrin); catalase (Fe-protoporphyrin+bile pigment hemochromogen). Some of the copper-containing proteins are: hemocyanin, turacin, polyphenol oxidase, ascorbic oxidase, hepatocuprein, and hemocuprein. The classical example of a prosthetic group that contains magnesium is chlorophyll. It is altogether probable that many other

enzymes contain a metallic element as a part of the complex molecule.

It is of interest to point out that the thioether linkage appears to be a characteristic of cytochrome c. We may represent the combination between the prosthetic group and protein as follows:



The sulfur bridge appears to be cystine.

A few examples of recently described protein complexes that contain a metallic element are given by Mann and Keilin (80) (hemocuprein); Dalton and Nelson (81) (tyrosinase); Kubowitz (82) (dehydrogenases); Agner (83) (catalase); Rawlinson (84) (hemoglobin); Jesserer and Lieben (85) (casein).

4. Other Protein-Complex Enzymes. Among the group of oxidation enzymes there are those that contain phosphoric acid in the prosthetic group. Presumably the phosphoric acid component of this molecule permits the prosthetic group to combine with an amino group of the protein molecule. It is quite possible that this combination takes place between the OH-group of phosphoric acid and the NH₂-group in the guanidino moiety of arginine yield-

^{*} This appears to be a tertiary cyclic nitrogen-containing base that is combined with the porphyrin in the 2- or 4- positions with the formation of a quarternary ammonium salt. See Theorell, H., Biochem. Z., 298, 242 (1938); Enzymologia, 6, 88 (1939).

acid with other amino groups is not excluded. Among this class of enzymes are: flavoproteins with prosthetic groups consisting of riboflavin phosphate, flavinadenine dinucleotide, and flavinadenine dinucleotide plus some as yet unknown groupings; pyridine proteins with a prosthetic group containing a mole each of adenine and nicotinic acid amide, 2 moles of pentose, and 2 moles of phosphoric acid; thiaminoprotein with diphosphothiamine as the prosthetic group (79).

5. Carbohydrate Proteins. Since it became recognized that many proteins contain carbohydrates (86), the carbohydrate proteins have been assigned an increasingly important rôle in biological phenomena and especially in immunology. Some of the carbohydrates or carbohydrate derivatives that have been isolated from proteins are: glucose, lactose, mannose, glusosamine, galacturonic acid, aldobionic acid, amylose, and various polysaccharides.

The mode of combination between the carbohydrate and protein may probably take place in a number of ways. Amino sugars and glycuronic acid probably combine by formation of a peptide linkage. It is doubtful that there are a sufficient number of α -amino groups in proteins to account for as much carbohydrate as 10 per cent (serum albumin). The ϵ -group of lysine and the NH₂-group in the guanidino moiety of arginine offer possible sites for such com-

¹ Meyer, K. (Cold Spring Harbor Symp. Quant. Biol., 6, 91 (1938)) classifies the hexosamine-containing compounds on the nature of the carbohydrate radical as follows:

A. Mucopolysaccharides

- I. Containing uronic acid
 - (a) Sulfate-free
 - 1. Vitreous humor, umbilical cord, synovial fluid, group A streptococcus
 - 2. Type I pneumococcus (?)
 - (b) Sulfate-containing
 - 1. chondroitinsulfuric acid
 - 2. mucoitinsulfuric acid (from gastric 'mucin' and cornea)
 - 3. heparin
- II. Neutral mucopolysaccharides of known composition
 - (a) chitin
 - (b) gastric polysaccharide
 - (c) bacterial polysaccharide
- B. Glycoproteins, containing neutral mucopolysaccharides of unknown composition
 - (a) ovomucoid-α (formerly called ovomucoid)
 - (b) ovomucoid-β (formerly called ovomucin)
 - (c) serum mucoid, serum glycoid
 - (d) globulins (egg white, serum albumin, thyroglobulin)
 - (e) pregnancy urine hormone

binations. If the carbohydrate is phosphorylated, combination with protein may take place by the formation of a -N-P= linkage.

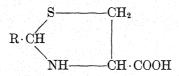
If the carbohydrate component is an aldose, we can imagine the following possibilities:

- (a) $R \cdot COOH + R' \cdot CHO \rightarrow R \cdot C : O \cdot (OC) \cdot R'$
- (b) $R \cdot NH_2 + R' \cdot CHOH \cdot R'' \rightarrow R \cdot NH \cdot CH(R') \cdot R''$

(c)
$$HC$$
— N HC — N $CH+R'\cdot CHOH\cdot R''\rightarrow R\cdot C$ — N R' — CH

The compounds formed in reactions (a) and (b) are easily hydrolyzed in the presence of acids and bases. Reaction (c) is a likelihood (87). Definite proof is, however, lacking.

Cysteine may combine with sugars. Schubert (88) has prepared such compounds in which the carbohydrate components were arabinose, glucose, mannose, and lactose. The combination takes place in a mole to mole ratio with the elimination of water. A compound of the following type is probably formed:



Since proteins contain cysteine, it is possible but not proven that some carbohydrates may be combined to proteins in this manner. It is evident that a great deal more work on the whole subject is necessary before we can consider our knowledge satisfactory.

6. Lipoproteins. Our knowledge of lipoproteins is less complete than that of carbohydrate proteins. It is altogether probable that such lipoproteins as lecithinoproteins exist (89). It is easily conceivable that basic proteins may form salts with fatty acids. Other possibilities are by ester or peptide linkage. Lecithin, sphingomyelin, and cephalin might possibly combine with proteins through

a —N—P= linkage. Cephalin might be combined as a peptide. It

is also conceivable that the fatty acid in sphingomyelin (attached

by means of a —CONH—linkage) might be replaced by a protein. Most of the above is speculation. Our knowledge of this subject is too fragmentary to warrant any precise statement.

7. Nucleoproteins. Many nucleoproteins are known. The protein component is usually basic and often a protamin or a histone. It is especially interesting that tobacco mosaic virus is a nucleoprotein. The combination between protein and nucleic acid is probably in the nature of a salt although, as indicated in the section dealing with phosphoric acid-containing prosthetic groups, the combination may take place in a manner similar to these. Another possibility is an ester linkage between the thiol group of the protein and phos-

phoric acid giving a —S—P=O linkage. Studies in this field have,

in recent years, been reported by Menzel and Heidelberger (90), Thompson and Dubos (91), Sevag and coworkers (92), and Carter and Hall (93). A review is given by Doinikova (94). When consideration is given to the fact that it is possible to introduce 20–30 phosphoryl groups into egg albumin (95), the possibility that all phosphoric acid-containing compounds may conceivably combine with proteins should be borne in mind. Electrophoresis studies on nucleoproteins have been carried out by Hall (107).

8. Other Protein Compounds. (a) Chromoproteins consist of pigmented prosthetic groups combined with proteins. Human hair contains leucokeratin and melanokeratin. Red hair contains rhodokeratin. The prosthetic groups can be separated by acid hydrolysis and salting out (96). The cells of the photosynthetic purple bacteria contain pigments combined with proteins for which the term photosynthein is suggested (97). Phyllochlorins are chlorophyllprotein compounds (98). The eggs of the lobster contain a carotenoid-protein pigment termed ovoverdin (99). The carotenoid component is an ester of astacene (4, 5, 4', 5'-tetraketo-\betacarotene) (100). The stability of the linkage between the prosthetic group and the protein is more similar to the yellow enzyme than to hemoglobin. The protein component has an albuminoid character. The molecular weight of ovoverdin is about 300,000 and its isoelectric point is 6.7. One mole of the prosthetic group is contained in a mole of ovoverdin. The carotenoid-proteins are of great importance especially in vision. Thus visual purple is a retinene-protein compound. The pigment component of the visual system of marine fishes is rhodopsin and that of fresh water fishes is porphyropsin (101). The carotenoids of chicken retina are astacene, xanthophyll, and an unidentified hydrocarbon (102). The manner in which these pigments are combined with proteins is not definitely known. The possibility exists that phosphoric acid may constitute the linkage.

(b) Compound Proteins. Globin-insulin has been reported (103). This compound is analogous to protamin-insulin and represents another example of a compound protein. Considerable work has been carried out to determine whether or not the proteins of blood serum exist as a mixture of individual molecules or as loosely bound complexes that are broken up on the addition of chemical reagents. McFarlane (104) found that horse serum contained about 80 per cent of a light fraction together with 20 per cent of a heavier fraction although the albumin globulin ratio, as ordinarily determined, was unity. The proportion of lighter to heavier molecules eventually became unity on dilution of the serum. A state of affairs approximating that in native serum could be obtained by starting out with mixtures of albumin and globulin and increasing the concentration. Eventually the proportion of the light to the heavy fraction was the same as in native serum. This suggests that there is a marked interaction between the component proteins of serum. The change in the sedimentation constants of serum proteins on addition of protamin (105) is also indicative of interaction between the components. All of this is suggestive that the proteins, particularly those occurring in native tissues and fluids, are probably reversibly dissociable systems, the equilibrium depending on the nature of the protein components and the dilution. When consideration is given to the fact that biological systems contain many components besides proteins (water, salts, lipids, carbohydrates, etc.) all of which may influence interreactivity, the true state of any protein component is still a matter of conjecture. The term "protein symplexes" has been suggested (106) for such more or less loosely defined complexes as polyproteins, polyosoproteins, lipoproteins, etc. The idea of protein complexes is not new. Hardy and Robertson (see page 775) both pointed out this possibility many years ago. The fact that proteins combine with such a large variety of compounds places them, from a biological standpoint, in the first rank of importance.

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CHAPTER XV

SOME THERMODYNAMICAL CONSIDERATIONS OF AMINO ACIDS, PEPTIDES, AND RELATED SUBSTANCES

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1. Thermal Data. Borsook, Huffman, Ellis, and coworkers have contributed, over a number of years, important thermal data from which thermodynamic data may be calculated. Most of these data were assembled in Table II, page 832. In order that all of their data may be available, Tables I, II, and III have been prepared. These tables include data that were not included in the original Table II plus more recent data. No recalculations have been made of the data in these tables.

All of the values of ΔH and ΔF as given in the earlier papers are subject to some revision on account of changes in the atomic weights of carbon and hydrogen and also for newer and better values for the heat of formation of water. These absolute errors will lead to no error in combining results to obtain values for ΔF and ΔH of reactions if the same data used are used in calculating the ΔH and ΔF of formation values in all cases.

In a series of studies from Gucker's laboratory, it has been shown that for non-electrolytes the limiting slope of the apparent molal volumes, expansibilities, heat capacities, and heat contents are linear functions of the first power of the concentration, rather than its square root, as in the case of electrolytes (12). These statements find support from the evidence that follows.

2. Molal Heat Capacities. (a) Sucrose. When the values for the apparent molal heat capacity of sucrose (13) are plotted against the concentration, the curve is almost a straight line. Values for Φ at the indicated temperatures can be calculated from the equations

$$\Phi = 145.87 + 1.950m - 0.1182m^2 (20^\circ)$$
 (1a)

$$\Phi = 151.20 + 1.325m - 0.0704m^2 (25^{\circ})$$
 (1b)

where m = molality.

Data Derived from Combustion of Amino Acids and Other Compounds TABLE I

Ref.	പയയപ്പെയിയയായിയിൽനെ സ്ത്രയയാല ഷപയയാനെ ക്യാവ്വ്വ സ്
$-\Delta F_f^*$, keal., mole ⁻¹	88.99 127.68 184.74 175.59 7.89 174.94
$\Delta U_{R/dT^1}$ mole ⁻¹ , deg. ⁻¹ cal.	(c) (c) (d) (d) (d) (d) (d) (d) (d) (d) (d) (d
ΔH_r^2 , keal., mole ⁻¹	221.76 134.81 135.85 240.01 189.58 260.57 283.49 129.31 129.31 157.94 194.83 376.93 37
$-\Delta H_R$, kcal., mole ⁻¹	663.74±0.21 386.55±0.16 273.58±0.16 273.58±0.16 273.58±0.16 450.63±0.17 450.96±0.19 382.57±0.19 382.57±0.19 555.06±0.20
$-\Delta U_B$, kcal., mole-1	664 48 ± 0.21 386, 386 ± 0.13 386, 386 ± 0.13 386, 386 ± 0.13 386, 10.14 460, 138 ± 0.16 460, 138 ± 0.19 383, 0.15 ± 0.19 383, 0.15 ± 0.19 383, 0.15 ± 0.19 555, 21 ± 0.20 555, 21 ± 0.20 555, 21 ± 0.20 557, 22 ± 0.13 666, 138 ± 0.13 666, 138 ± 0.20 537, 22 ± 0.13 537, 22 ± 0.13 537, 22 ± 0.13 537, 22 ± 0.13 537, 23 ± 0.13 537, 2
$-\Delta U_B$, keal., mole $^{-1}$	665.01±0.16 386.65±0.11 386.65±0.11 271.73±0.10 271.73
Density	4.5.5.5.5.4.4.6.5.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1.1
Compound	Adenine d-Alamine d-Alamine d-Alamine d-Alamine All-Antoin

 $-\Delta U_B$ =energy evolved in the isothermic bomb process; ΔU_R =energy evolved corrected to condition where reactants and products are each at a pressure of one atmosphere; ΔH_R^0 =heat of formation from the elements; $\Delta U_{R/dT}$ =temperature coefficient of ΔU_R . (Definitions given in ref. (1).)

1 All minus values are for $\Delta U_{R/dT}$.

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 ${\bf TABLE~II} \\ Molal~Entropies~of~Amino~Acids~and~Related~Compounds~in~Cal.~Degree^{-1}~Mole^{-1} \\$

Substance	S_{90}°	S ₉₀ °_298.1°	$S_{298.1}$ °	Ref.
Adenine	11.16	24.90	36.1	3
d-Alanine	8.88	22.77	31.6	4
dl-Alanine	8.84	22.80	31.6	6
Allantoin	14.18	32.39	46.6	3
dl-Alanylglycine	15.84	35.15	51.0	7
Alloxan	13.15	31.43	44.6	3
d-Arginine	17.52	42.52	59.9	6
l-Asparagine	11.90	29.84	41.7	4
l-Asparagine, hyd.	13.57	37.46	51.0	4
l-Aspartic acid	12.21	29.28	41.5	4
dl-Citrulline	18.15	42.65	60.8	1
Creatine	13.31	31.99	45.3	4
Creatine, hydrate	16.39	39.62	56.0	1
Creatinine	13.38	26.66	40.0	4
l-Cysteine	11.71	28.85	40.6	5
l-Cystine	18.99	49.51	68.5	5
β - β' -Dithiolactic acid	20.18	45.28	65.5	5
d-Glutamic acid	13.00	32.73	45.7	4
Glutamic acid hydrochloride	18.39	40.94	59.33	2
Glycylglycine	13.60	31.80	45.4	7
Guanidine carbonate	22.31	48.28	70.59	2
Guanine	10.77	27.55	38.3	3
Hippuric acid	18.48	38.72	57.2	7
Hippurylglycine	24.14	51.04	75.2	7
Hypoxanthine	10.89	23.87	34.8	3
d(l+)—Lactic acid	10.43	23.87	34.30	2
l(d-)-Lactic acid	10.26	23.75	34.00	2
dl-Leucine	13.91	35.61	49.5	6
dl-Leucylglycine	19.71	45.50	67.2	7
dl-Ornithine	13.16	33.08	46.2	1
Ornithine dihydrochloride	21.31	48.94	70.25	2
l-Proline	13.40	27.38	40.8	1
Taurine	10.56	26.19	36.8	1
β-Thiolactic acid	11.88	27.86	54.7	5
l-Tyrosine	15.43	37.60	53.0	6
Uric acid	12.01	29.42	41.4	3
Xanthine	11.68	26.80	38.5	3

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Table III

Thermal Data for Amino Acids and Other Compounds¹

Compound	Heat of comb. at const. π	$\Delta H^{\circ}_{298.1}$ ° cal./mole	$\Delta S_{298,1}^{\circ}$ E. U.	$\Delta F^{\circ}_{298.1}^{\circ}$ cal./mole	Ref
	cal./mole	01 800	100.0	70,420	
Adenine	663,740	21,760	-163.2	- 88,800	5
d-Alanine	387,200	-134,600	-153.5	-89,440	3
dl-Alanine	386,550	-135,260	-153.7	-39,440 $-107,470$	1
Allantoin	409,550	-172,350	-217.6		1
Alloxan	273,580	-240,010	-191.7	-182,880	3
d-Arginine	893,480	-150,160	-307.5	- 58,490	5
l-Asparagine	463,100	-187,100	-207.7	-125,200	5 5
l-Asparagine hyd.	459,400	-259,100	-254.2	-183,300	
l-Aspartic acid	384,700	-231,300	-193.9	-173,500	5
Creatine	555,200	-129,200	-218.2	- 64,100	5
Creatinine	563,200	-52,800	-167.6	- 2,900	5
<i>l</i> -Cysteine	532,420	-127,660	-152.3	-82,260	2
l-Cystine	998,170	-251,520	-286.1	-166,230	2
β - β' -Dithiolactic acid	946,800	-231,770	-212.0	-168,570	2
Fumaric acid		-194,880	-126.3	-157,230	4
α -d-Glucose	-	-305,730	-291.9	-218,720	4
α -d-Glucose, hyd.		$-376,520\dagger$	-338.0 †	$-275,760\dagger$	4
β -d-Glucose		$-304,230\dagger$	-288.2 †	$-218,320\dagger$	4
d-Glutamic acid	542,200	-236,400	-222.3	-170,200	5
Glycine	232,570	-126,690	-126.6	-88,950	3
Guanine	596,890	-45,090	-185.6	10,220	1
Hypoxanthine	580,200	-27,630	-150.5	17,150	. 1
d-Leucine	856,010	-153,470	-233.6	- 83,830	3
l-Leucine	855,980	-153,490	-233.6	- 83,860	3
dl-Leucine	855,230	-154,250	-233.6	-84,610	3
Maleic acid		-189,450	-127.9	-151,320	4
Succinic acid		-225,660	-155.2	-179,360	4
β-Thiolactic acid	511,500	-111,940	- 99.7	-82,220	2
l-Tyrosine	1,058,340	-165,540	-227.4	-97,750	3
Uric acid	458,840	-148,980	-193.0	-91,460	1
Xanthine	516,020	- 91,810	-171.4	-40,730	1

[†] Calculated.

A test of the third law of thermodynamics has again been made by Huffman, H. M., J. Amer. Chem. Soc., 62, 1009 (1940) by the use of urea. The data are:

$$\begin{array}{c} {\rm CO_2}(g) + 2{\rm NH_3}(g) = {\rm H_2O}(g) + {\rm CO\,(NH_2)_2\,(s)}; \, \Delta F_{298} = 290 \\ {\rm H_2O}(g) = {\rm H_2 + \frac{1}{2}\,O_2}; \, \Delta F = 54\,,638 \\ {\rm C\,\,(graph) + O_2 = CO_2(g)}; \, \Delta F = -94\,,239 \\ {\rm N_2 + 3H_2 = 2NH_3}; \, \Delta F = 7820 \end{array}$$

C (graph) $+2H_2+N_2+1/2$ $O_2 = CO(NH_2)_2(s)$; $\Delta F = -47,131$ This result is only 80 calories higher than the third law value and is less than the uncertainty in the $T\Delta S$ term due to the uncertainty in the entropy of urea.

Values for the partial molal heat capacities of solute and solvent (sucrose in aqueous solution) can be calculated from the equations

$$\overline{C}_{p2} = 145.87 + 3.900m - 0.3546m^2$$
) (At 20° in (2a)

$$\overline{C}_{p1}^{\circ} - \overline{C}_{p1} = 0.018016(1.950m^2 - 0.2364m^3)$$
 $\left. \begin{array}{c} \text{cal.}_{20}/\text{deg.} \end{array} \right)$ (2b)

$$\overline{C}_{p2} = 151.20 + 2.650m - 0.2112m^2$$
) (At 25° in (3a)

$$\overline{C}_{p1}^{\circ} = \overline{C}_{p1} = 0.018016(1.325m^2 - 0.1408m^3)$$
 cal_{.25}/deg.) (3b)

(b) Urea. The curve showing the relation between the apparent molal heat capacities and the square root of the molality of aqueous urea solutions (14) is described by an equation of the type

$$\Phi = \Phi^{\circ} + am^{1/2} + bm - cm^{3/2} \tag{4}$$

The apparent molal heat capacity is calculated from the equation

$$\Phi(C_{p2}) = \left\lceil \frac{1000}{m} + M_2 \right\rceil s - \frac{1000}{m} \tag{5}$$

where $M_2 = 60.057$ (mol. wt. urea). s, the specific heat capacity, is given in calorie units at the experimental temperature. Φ , the apparent molal heat capacity, is expressed in cal./degree/mole. The partial molal heat capacities of solute and solvent can be calculated from the equations

$$\overline{C}_{n2} = \Phi C_{n2}^{\circ} + 3/2am^{1/2} + 2bm + 5/2cm^{3/2},\tag{6}$$

$$\overline{C}_{v1} = \overline{C}_{v1}^{\circ} - 10^{-3} M_1 (1/2am^{3/2} + bm^2 + 3/2cm^{5/2})$$
(7)

Since M_1 , the molecular weight of water, is 18.0156 equation (7) becomes

$$\overline{C}_{p1} = 18.0156 \left[1 - 10^{-3} \left(\frac{1}{2am^{3/2} + bm^2 + \frac{3}{2cm^{5/2}}} \right) \right]$$
 (7a)

The coefficients of equation (4) are used in computing values in the above equations.

¹ In later work (16) these equations were changed to $\frac{\overline{C}_{p2}^{\circ}}{\overline{C}_{p1}^{\circ}} = 151.50 + 2.260m - 0.1398 \ m^{2},$ $\overline{C}_{p1}^{\circ} - C_{p1} = 0.018016 \ (1.130 \ m^{2} - 0.0932 \ m^{3})$

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(c) Glycine and Glycolamide (15). The apparent molal heat capacities of these compounds may be calculated by use of the equation

 $\Phi C_{p2} = \Phi^{\circ} C_{p2} + am + bm^2 \tag{8}$

The effect of m^2 is negligible at low molalities, M. The equations for glycine and glycolamide are given in Table IV.

Table IV

Equations for the Apparent Molal Heat Capacities of Glycine and Glycolamide and the Partial Molal Heat Capacities of Solute and Solvent at 5°, 25°, and 40°

	Glycine	Glycolamide
$5^{\circ} \begin{cases} \Phi C_{p2} = \\ \overline{C}_{p2} = \\ \overline{C}_{p1} - \overline{C}_{p1} = \end{cases}$	$\begin{array}{r} -4.30 + 9.01m - 1.25m^2 \\ -4.30 + 18.02m - 3.75m^2 \\ 0.1623m^2 - 0.0450m^3 \end{array}$	$26.66+2.20m-0.15m^2$ $26.66+4.40m-0.45m^2$ $0.0396m^2-0.0054m^3$
$25^{\circ} \begin{cases} \Phi \underline{C}_{p2} = \\ \overline{C}_{p2} = \\ \overline{C}_{p1}^{\circ} - \overline{C}_{p1} = \end{cases}$	$8.83 + 4.58m - 0.47m^{2} \\ 8.83 + 9.16m - 1.41m^{2} \\ 0.0825m^{2} - 0.0169m^{3}$	$35.69 + 0.72m - 0.03m^2 \ 35.69 + 1.44m - 0.09m^2 \ 0.0130m^2 - 0.0011m^3$
$40^{\circ} \begin{cases} \Phi C_{p2} = \\ \overline{C}_{p2} = \\ \overline{C}_{p1}^{\circ} - \overline{C}_{p1} = \end{cases}$	$13.74 + 3.27m + 0.28m^{2} 13.74 + 6.54m - 0.83m^{2} 0.0589m^{2} - 0.0101m^{3}$	$39.15 + 0.30m$ $39.15 + 0.60m$ $0.0054m^2$

(Gucker Jr., F. T., Ford, W. L., and Moser, C. E., J. Phys. Chem., 43, 153 (1939); Gucker Jr., F. T., and Ford, W. L., J. Phys. Chem., 45, 309 (1941).)

The molal heat capacity of solid glycine at 25° is 23.9 cal. per degree (25). The apparent molal heat capacity at infinite dilution is 15.2 cal. per degree less at 25°. This difference varies enormously with temperature. It is twice as great at 5° and only two-thirds as large at 40°.

(d) dl- α -Alanine, β -Alanine, and Lactamide (43). The following are the equations for the apparent molal heat capacities of dl- α -alanine, β -alanine, and lactamide and the partial molal heat capacities of solute and solvent at 5, 25, and 40°:

$$dl\text{-}\alpha\text{-}Alanine$$

$$5^{\circ} \begin{cases} \Phi C_{p2} = 23.75 + 3.70m - 0.20m^{2} \\ \overline{C}_{p2} = 23.75 + 7.40m - 0.60m^{2} \\ \overline{C}_{p1}^{\circ} - \overline{C}_{p1} = 0.666m^{2} - 0.0072m^{3} \end{cases}$$

$$\Phi C_{p2} = 33.69 + 1.48m + 0.09m^{2} \\ \overline{C}_{p2} = 33.69 + 2.96m + 0.27m^{2} \\ \overline{C}_{p1}^{\circ} - \overline{C}_{p1} = 0.0267m^{2} + 0.0032m^{3} \end{cases}$$

$$\Phi C_{p2} = 36.66 + 1.99m - 0.22m^{2} \\ \overline{C}_{p2} = 36.66 + 3.98m - 0.66m^{2} \\ \overline{C}_{p1}^{\circ} - \overline{C}_{p1} = 0.0359m^{2} + 0.0079m^{3} \end{cases}$$

$$\beta\text{-Alanine}$$

$$5^{\circ}\begin{cases} \Phi C_{p2} = 4.04 + 6.67m - 0.86m^{2} + 0.05m^{3} \\ \overline{C}_{p2} = 4.04 + 13.34m - 2.58m^{2} + 0.20m^{3} \\ \overline{C}_{p1} - \overline{C}_{p1} = 0.1200m^{2} - 0.0310m^{3} + 0.0027m^{4} \end{cases}$$

$$25^{\circ}\begin{cases} \Phi C_{p2} = 18.27 + 2.64m - 0.13m^{2} \\ \overline{C}_{p2} = 18.27 + 5.28m - 0.39m^{2} \\ \overline{C}_{p1} - \overline{C}_{p1} = 0.0475m^{2} - 0.0047m^{3} \end{cases}$$

$$\Phi C_{p2} = 23.34 + 2.12m - 0.10m^{2} \\ \overline{C}_{p1} - \overline{C}_{p1} = 0.0381m^{2} - 0.0036m^{3} \end{cases}$$

$$\text{Lactamide } [\text{CH}_{3} \cdot \text{CH}(\text{OH}) \cdot \text{CONH}_{2}]$$

$$5^{\circ}\begin{cases} \Phi C_{p2} = 52.44 - 0.43m + 0.05m^{2} \\ \overline{C}_{p1} - \overline{C}_{p1} = -0.0078m^{2} + 0.0016m^{3} \end{cases}$$

$$\Phi C_{p2} = 53.38 - 0.48m + 0.02m^{2} \\ \overline{C}_{p1} - \overline{C}_{p1} = -0.0087m^{2} + 0.0006m^{3} \end{cases}$$

$$\Phi C_{p2} = 60.92 - 0.57m + 0.02m^{2} \\ \overline{C}_{p1} - \overline{C}_{p1} = -0.0087m^{2} + 0.0006m^{3} \end{cases}$$

$$\Phi C_{p2} = 60.92 - 1.14m + 0.06m^{2} \\ \overline{C}_{p1} - \overline{C}_{p1} = -0.0103m^{2} + 0.0007m^{3} \end{cases}$$

The decrease in heat capacity caused by the formation of free ions is of interest in connection with the zwitterion structure of the amino acids. In the case of water and its ions this is 50.6 cal. per degree per mole (12). Edsall's calculations (15) show for amino acids:

H⁺+H₂N·R·COO⁻=+H₃N·R·COO⁻ (
$$\Delta C_{p1}$$
= -7(5°) and +25 cal. deg.⁻¹ mole⁻¹)

The reaction can be represented as the sum of the two reactions

$$\begin{aligned} &H_2N\cdot R\cdot COOH=^+H_3N\cdot R\cdot COO^- & \text{ and } \\ &H_2N\cdot R\cdot COO^-+H^+=H_2N\cdot R\cdot COOH & \end{aligned}$$

Assuming that the change of heat capacity is about the same for the ionization of propionic acid (26), $\Delta C_{p3} = 35$ (5°) and 39 (40°) cal. deg.⁻¹ mole⁻¹. By subtracting, ΔC_{p2} (change from uncharged acid to zwitter ion) is -42 (5°) and -14 (40°) cal. deg.⁻¹ mole⁻¹.

3. Heat of Dilution. (a) Sucrose (16). The relation of the heat of dilution of sucrose to its concentration and at the indicated temperatures is given by the equations

$$\Delta H/\Delta m = 128.9 = d\Phi H_2/dm (20^\circ)
\Delta H/\Delta m = 140.2 = d\Phi H_2/dm (30^\circ)
M=0.2$$
(9a)

(b) Urea (17). The heat of dilution of urea may be calculated from the equation

$$\Delta H/\Delta m = -85.87 + 12.88m - 0.795m^2 \tag{10}$$

(c) Glycine. Similar values for glycine¹ (18) may be calculated from

$$\Delta H/\Delta m = -106.8 + 36.60m - 5.09m^2 \tag{11}$$

The heat of dilution curve for glycolamide is more linear than that for glycine. It is evident from the above that glycine, glycolamide, and urea absorb heat on dilution while sucrose liberates heat.

4. Heat Content. (a) Sucrose (16). Integration of equation (10) yields the following for the apparent molal heat content of sucrose below 0.2 m

$$\Phi H_2 = \Phi H_2^{\circ} + 128.9m \ (20^{\circ}) \tag{12a}$$

$$\Phi H_2 = \Phi H_2^{\circ} + 140.2m \ (30^{\circ}) \tag{12b}$$

where ΦH_2° is the apparent molal heat content in the infinitely dilute solution.

The apparent relative molal heat content is given by

$$\Phi L_2 = \Phi H_2 - \Phi H_2^{\circ} \tag{13}$$

For sucrose at the indicated temperatures and concentrations (16)

$$\Phi L_2 = 128.9m (20^\circ)
\Phi L_2 = 140.2m (30^\circ)
(13a)$$
(13b)

$$\Phi L_2 = 140.2m \ (30^{\circ})$$
 (13b)

$$\Phi L_2 = 134.6m - 7.05m^2 (25^\circ, \text{ and } m \le 2.2m)$$
 (13c)

The latter equation is based on values obtained from the literature. The apparent relative molal heat content of the solute is related to the relative heat content of the solution, L, by the equation

$$\Phi L_2 = \frac{L - 55.51\overline{L}_1^{\circ}}{m} \tag{14}$$

Since \overline{L}_i° is zero

$$L = m\Phi L_2 \tag{15}$$

Differentiating with respect to m gives the relative partial molal heat content of the solute as

$$\overline{L}_2 = \frac{\partial L}{\partial m} = \Phi L_2 + m \frac{\partial \Phi L_2}{\partial m} \tag{16}$$

Substituting the values ΦL_2 and $(\partial \Phi L_2/\partial m)$ into this equation gives

¹ See (919) and Wallace, W. E., Offutt, W. F., and Robinson, A. L., J. Amer. Chem. Soc., 65, 347 (1943).

$$\overline{L}_2 = 257.8m (20^\circ)
\overline{L}_2 = 280.4m (30^\circ)
(16a)$$
(16b)

$$\overline{L}_2 = 280.4m \ (30^\circ) \int_{-\infty}^{\infty} (m \ge 0.2)$$
 (16b)

Since

$$L = m\Phi L_2 = 55.51\overline{L}_1 + m\overline{L}_2$$

$$\overline{L}_1 = \frac{m(\Phi L_2 - \overline{L}_2)}{55.51} \tag{17}$$

$$\overline{L}_{1} = -2.322m^{2} (20^{\circ})
\overline{L}_{1} = -2.525m^{2} (30^{\circ})
(17a)$$
(17a)
(17b)

$$\overline{L}_1 = -2.525m^2 (30^\circ)$$
 (17b)

From the above it is seen that the apparent molal heat capacity and the heat of dilution of sucrose is a linear function of m over the range studied.

(b) Urea (17). The equation for the apparent relative molal heat content of urea, obtained by integrating equation (10) is given by

$$\Phi L_2 = -85.87m + 6.815m^2 - 0.4569m^3 + 0.01471m^4$$
 (18)

that of glycine (18), obtained by integrating equation (11) by

$$\Phi L_2 = -106.8m + 18.30m^2 - 1.70m^3$$
 (see page 839) (19)

Sturtevant (19) finds

$$\Phi H - \Phi H^{\circ} = \frac{-485m}{1 + 0.395m} - 23.7m^{2} \tag{20}$$

For glycolamide (17), (CH₂OH·CONH₂), the isomer of glycine,

$$\Phi L_2 = -46.00m + 3.52m^2 - 0.134m^3 \tag{21}$$

The equations for the apparent relative molal heat contents as functions of the temperature between 5 and 40° are:

(Glycine)

$$10^{3}(\Phi L_{2} - \Phi L_{2}^{"}) = (4570m - 470m^{2})(t - 25) - (73.0m - 12.1m^{2})(t - 25)^{2} + (1.30m - 0.26m^{2})(t - 25)^{3}$$
(22)

(Glycolamide)

$$10^{3}(\Phi L_{2} - \Phi L_{2}^{"}) = (720m - 30m^{2})(t - 25) - (24.0m - 1.9m^{2})(t - 25)^{2} + 0.48m - 0.03m^{2})(t - 25)^{3}$$

$$(23)$$

5. Molal Volumes. (a) Urea. The apparent molal volume of urea is a linear function of the volume concentration, c, at 30° and up to 3 molar at 25° (20). At the two temperatures this relation is expressed by the equations

$$\Phi V_2 = 44.224 + 0.1319c (25^\circ) \le 3m$$
 (24a)

$$\Phi V_2 = 44.546 + 0.1087c (30^{\circ})$$
 (24b)

The corresponding density equations are:

$$d = 0.997074 + 0.015964c - 1.315 \times 10^{-4} c^{2} (25^{\circ})$$
 (25a)

$$d = 0.995673 + 0.015705c - 1.082 \times 10^{-4} c^2 (30^{\circ})$$
 (25b)

The density of solid urea is 1.329 gm./ml. at 25° and its molal volume is 45.19 ml.

(b) Glycine and Glycolamide (15). Equations for the apparent molal volumes of glycine and glycolamide and the partial molal volumes of solute and solvent at 25° are given in Table V.

TABLE V

Equations for the Apparent Molal Volumes of Glycine and Glycolamide and the Partial Molal Volumes of Solute and Solvent at 25°

Glycine	Glycolamide
$\Phi V_2 = 43.199 + 0.8614c$	$\Phi V_2 = 56.168 + 0.1294c$
$\overline{V}_2 = 43.199 + \left[\frac{2000 - 43.199c}{1000 + 0.8614c^2} \right]$	$\overline{V}_2 = 56.168 + 0.2588c - 0.00727c^2$
$\overline{V}_1 = \frac{18069.1}{1000 + 0.8614c^2}$	$\overline{V}_1 = 18.0691 - 0.002338c^2$

At infinite dilution V_2 for glycine is 12.96 ml. less than that for glycolamide. This value may be taken as the electrostriction of the solvent. The molal volume of solid glycolamide is 54.01 ml. and that of glycine is 46.71 ml. The difference, 7.30 ml. shows the much closer packing in the crystal lattice of glycine, due to the electrostatic forces. In an infinitely dilute solution, the apparent or partial molal volume of glycolamide is 56.16 or 2.15 ml. larger than that of the solid. Assuming 10 per cent increase in volume (23) on melting, the estimated apparent molal volume is 59.4 ml. if it formed a perfect solution. The difference (59.4-56.16=3.2) is due to electrostriction. In the case of glycine, the limiting value of the partial molal volume is 3.51 ml. less than the molal volume of the solid. This indicates that the electrostrictive effect upon the solvent is even greater than that in the crystal lattice.

The densities of glycine and glycolamide solutions at 25° are given by

$$d = 0.997074 + 0.031996c - 8.589 \times 10^{-4} c^2 \text{ (glycine)}$$
 (26a)

$$d = 0.997074 + 0.019065c - 0.000129 c^2$$
 (glycolamide) (26b)

where c = molarity.

The following are the equations for the densities and apparent molal volumes of dl- α -alanine, β -alanine, and lactamide and the partial molal volumes of solute and solvent at 25° (43):

$$\begin{array}{c} dl\text{-}\alpha\text{-}\text{Alanine} \\ d=0.997074+0.028663c-5.73\times 10^{-4}c^2 \\ \Phi V_2=60.609+0.5731c \\ \overline{V}_2=60.609+1.1462c-0.03473c^2 \\ \overline{V}_1=18.0691-0.01035c^2 \end{array}$$

$$\begin{array}{c} \beta\text{-Alanine} \\ d = 0.997074 + 0.030543c - 7.147 \times 10^{-4}c^2 \\ \Phi V_2 = 58.723 + 0.7168c \\ \overline{V}_2 = 58.723 + 1.4336c - 0.04209c^2 \\ \overline{V}_1 = 18.0691 - 0.01340c^2 \end{array}$$

Lactamide
$$d = 0.997074 + 0.015802c - 1.684 \times 10^{-4}c^2$$
 $\Phi V_2 = 73.508 + 0.0169c$ $\overline{V}_2 = 73.508 + 0.0338c - 0.00124c^2$ $\overline{V}_1 = 18.0691 - 0.000305c^2$

The apparent molal volumes in each case is a linear function of the molarity. The difference between the limiting values for α -alanine and lactamide is 12.90 ml. and between β -alanine and lactamide the value is 14.78 ml. These are the values for the electrostriction of the solvent.

6. Coefficient of Expansibility. The equations for the densities may, by suitable calculations, (10) be used to calculate the mean coefficient of expansibility. For aqueous *urea* (10) solution at 27.5° the equation is

$$\alpha_{27.5} = (281.2 + 52.0c - 4.71c^2) \times 10^{-6}$$
 (27)

The apparent molal expansibility at 27.5° is given by

$$\Phi E_2 = 0.0645 - 0.00468c \tag{28}$$

The partial molal expansibilities of solute and solvent, which are the temperature coefficients of the corresponding partial molal volumes, in the case of aqueous urea solutions at 27.5°, are given by the equations

$$\overline{E}_2 = 0.0645 - 0.00936c + 0.00208c^2 \tag{29}$$

$$\overline{E}_1 = 0.00508 + 0.0000846c^2 - 1 \times 10^{-8} c^4$$
 (30)

The method developed for calculating the coefficient of expansi-

bility of urea solutions may be used for any solution for which the density is known as a function of the molarity at two different temperatures. The apparent molal expansibility of urea is about as great as that of a uni-univalent electrolyte, although it is a linear function of the first power and not the square root of the molarity.

7. Thermodynamic Considerations. For glycine (18), the change in free energy on dilution to a solution of unit activity is given by

$$\Delta F = -RT \ln \gamma_m = -525 \text{ cal.} \tag{31}$$

when the solubility of glycine is taken as 3.33 m at 25° and γ =0.729 in the saturated solution. The heat of solution of solid glycine in the saturated solution is

$$\Delta H = \overline{L}_2 \text{ (sat.)} - \overline{L}_2(s) = -354 + 3765 = 3411 \text{ cal.}$$
 (32)

whence the entropy of solution in the saturated solution is

$$\Delta S = 3411/298.16 = 11.4 \text{ E.U.}$$
 (33)

The following thermodynamic values have been calculated for the hypothetical one molal aqueous glycine solution: ΔH° (kcal./mole) = -122.51; ΔF° (kcal./mole) = -89.05; ΔS° (E. U.) = -122.2 S(E. U.) = 40.5. For solid glycine the corresponding thermodynamic values are: -126.27; -88.52; -126.6; 26.1.

8. **Résumé.** A review of the apparent molal heat capacity data for methyl alcohol, ethyl alcohol, n-propyl alcohol, glycol, glycerine, and acetone shows that, in contradiction to the behavior of all electrolytes and of sucrose, urea, glycine, and mannite, the apparent molal heat capacities of all these solutes except n-propyl alcohol decrease with increasing concentration. It is to be noted that the apparent molal heat capacity of sucrose is more nearly a linear function of the first power of the molarity than of its square root. In the case of urea, the change is not linear with respect to molarity, as is the apparent molal volume, or with respect to m, N_2 , or the square root of any of these quantities. The simplest type of curve is obtained by plotting $C^{1/2}$. The apparent molal heat capacity of urea changes less with concentration than that of a uni-univalent electrolyte. There is no relationship between the apparent molal heat capacity and any function of the concentration such as the linear relationship between $\Phi(C_{n2})$ and $m^{1/2}$ which Randall and Rossini (11) have shown to hold for most electrolytes.

Urea, glycine, and glycolamide absorb heat upon dilution while sucrose liberates heat. In case of all of these solutes, the apparent relative molal heat content is a linear function of the concentration as would be predicted by the theory of Fuoss (12). The theory predicts that all solutes when dissolved in water (dilute solution) should liberate heat and that the slope should be greater the larger the dipole moment of the solute particle. Apparently some other factor besides the dipole moment enters.

9. Ionization of Weak Electrolytes. Reference has already been made in Section 2 to the change in molal heat capacity due to the formation of zwitter ions from α -amino acids. Harned and Owen (27) have made use of the thermodynamic ionization constants of amino acids for the purpose of calculating ΔF° , ΔH° , ΔS° , and ΔC_{p}° . The ionization constants may be expressed with an average accuracy of the order of 0.002 in log K by the equation of Harned and Embree (28)

$$\log K = \log K_{\theta} - p(t - \theta)^2 \tag{34}$$

where K_{θ} is the value of K at its maximum, θ is the temperature at which K is a maximum, and p has a value of 5×10^{-5} deg.⁻².

The heat content and heat capacity changes of the ionization reaction at unit activities are given by the equations

$$\Delta H^{\circ} = -4.575 \times 10^{-4} \ T^{2}(t - \theta) \tag{35}$$

$$\Delta C_p^{\circ} = -4.575 \times 10^{-4} \ T(T + 2(t - \theta))$$
 (36)

These equations are derived by differentiating equations (34). The free energy and entropy changes are obtained from the customary thermodynamic equations. For the ionization of water, the equation

$$\log K_w = -4787.3/T - 7.1321 \log T - 0.010365T + 22.801$$
 (37)

may be used for temperatures from 10-35°. Thermodynamic values for some of the amino acids and related fatty acids are given in Table VI.

Inspection of Table VI shows that no simple inference can be drawn from the values of ΔF° , ΔH° , and ΔS° . On the other hand, $\Delta C_{\mathfrak{p}}^{\circ}$ shows some interesting regularities. The results may be arranged in three groups, corresponding to the type reactions,

$$\begin{array}{ccc} \mathrm{HA} + \mathrm{H}_2\mathrm{O} \rightleftarrows \mathrm{A}^- + \mathrm{H}_3\mathrm{O}^+ & \Delta C_p^{\circ} \simeq -41 \\ ^+\mathrm{NH}_3 \cdot \mathrm{R} \cdot \mathrm{COOH} + \mathrm{H}_2\mathrm{O} \rightleftarrows^+\mathrm{NH}_3 \cdot \mathrm{R} \cdot \mathrm{COO}^- + \mathrm{H}_3\mathrm{O}^+ & \Delta C_p^{\circ} \simeq -36 \\ \mathrm{NH}_2 \cdot \mathrm{R} \cdot \mathrm{COO}^- + \mathrm{H}_2\mathrm{O} \rightleftarrows^+\mathrm{NH}_3 \cdot \mathrm{R} \cdot \mathrm{COO}^- + \mathrm{OH}^- & \Delta C_p^{\circ} \simeq -22 \end{array}$$

Table VI

Parameters of Equation (34) and Derived Thermochemical Quantities

Electrolyte	Temp.	θ	$-\log K_{\theta}$	- log K25	ΔF°_{25}	ΔH° 25	ΔC° _{p25}	ΔS°25
Water				13.996	19090	13480	-42.5	-18.8
Formic acid	0-50	24.7	3.752	3.752	5117	-12	-40.7	-17.2
Acetic acid	0-45	22.6	4.756	4.756	6486	-98	-41.3	-22.1
Propionic acid	0-45	20.9	4.873	4.874	6647	-167	-41.8	-22.9
Butyric acid	0-45	8.0	4.803	4.817	6569	-691	-45.3	-24.4
Glycine (K_A)	10-45	53.9	2.309	2.351	3206	1175	-32.8	- 6.8
(K_B)	10-40	93.0	3.988	4.219	5754	2765	-22.1	-10.0
dl -Alanine (K_A)	0-50	44.8	2.330	2.350	3205	805	-35.3	- 8.1
(K_B)	0-50	88.0	3.933	4.132	5635	2561	-23.5	-10.3
dl - α -Amino- n -butyric acid (K_A)	0-50	32.6	2.285	2.288	3120	309	-38.6	- 9.4
(K_B)	0-50	95.2	3.923	4.169	5686	2854	-21.5	- 9.5
dl - α -Amino- n -valeric acid (K_A)	0-50	38.6	2.307	2.316	3159	553	-37.0	- 8.7
(K_B)	0-50	92.8	3.965	4.195	5721	2756	-22.2	-10.0
dl-Norleucine (KA)	0-50	38.8	2.323	2.332	3182	561	-36.9	- 8.8
(K_B)	0-50	89.4	3.958	3.165	4316	2618	-23.1	- 5.7
α -Aminoisobutyric acid (K_A)	0-50	38.8	2.349	2.359	3217	561	-36.9	- 8.9
(K_B)	0-50	75.0	3.667	3.792	5172	2033	-27.0	-10.5
dl-Valine (KA)	0-50	27.0	2.287	2.287	3119	81	-40.1	-10.2
(K_B)	0-50	97.0	4.022	4.281	5838	894	-34.7	-12.4
dl -Leucine (K_A)	0-50	35.4	2.324	2.329	3176	423	-37.8	- 9.2
(K_B)	0-50	93.0	4.022	4.253	5800	2765	-22.1	-10.2
dl-Isoleucine (KA)	0-50	32.4	2.317	2.320	3164	301	-38.6	- 9.6
(<i>K_B</i>)	0-50	93.8	4.009	4.246	5791	2797	-21.9	-10.0
Acetic acid in:								
10 per cent methyl alcohol	0-40	27.0	4.904	4.905	6690	81	-40.1	-22.2
20 per cent methyl alcohol	0-40	31.5	5.079	5.081	6930	264	-38.9	-22.4
20 per cent dioxane	0-50	24.6	5.293	5.292	7216	-16	-40.8	-24.3
45 per cent dioxane	0-50	15.26	6.305	6.307	8600	-394	-43.3	-30.2
70 per cent dioxane	0-50	10.42	8.313	8.321	11347	-594	-44.6	-40.1

(Harned, H. S., and Owen, B. B., Chem. Rev., 25, 31 (1939).)

The ionization of water may be used for comparing all of the results with a common reference reaction:

$$H_2O + H_2O \rightleftharpoons OH^- + H_3O^+$$
 $\Delta C_n^{\circ} = -42.5$

Subtracting the above three reactions in turn from the water reaction and including the first of them, we obtain

(a)	$\mathrm{HA} + \mathrm{H_2O} \rightleftharpoons \mathrm{A}^- + \mathrm{H_3O}^+$	ΔC_p ° $\simeq -41$
(b)	A ⁻ +H ₂ O⇔HA+OH ⁻	$\Delta C_p^{\circ} \simeq 0$
(c) +N	$VH_3 \cdot R \cdot COO^- + H_2O \rightleftharpoons + NH_3 \cdot R \cdot COOH + OH^-$	$\Delta C_p^{\circ} \simeq -5$
(d) +N	$NH_3 \cdot R \cdot COO^- + H_2O \rightleftharpoons NH_2 \cdot R \cdot COO^- + H_3O^+$	$\Delta C_p^{\circ} \simeq -20$

By subtracting reaction (c) from reaction (d) the reaction

(e) $NH_3 \cdot R \cdot COOH + OH \rightarrow NH_2 \cdot R \cdot COO + H_3O + \Delta C_p \simeq -15$ is obtained

From the above it is evident that reaction (a), which forms ions from neutral molecules, leads to the greatest change in heat capacity. Reaction (b) produces practically no change in heat capacity. Reactions (c) and (d) which are of the same electrical type as reaction (a) give rise to ΔC_p° values of a different magnitude than reaction (a). It is evident from the above discussion that the magnitude of ΔC_p ° depends upon both the chemical and electrical type of the reaction. It is also of interest to note in Table VI that ΔC_{p} ° for the ionization of acetic acid in certain aqueous mixed solvents is very nearly the same as that in water, although the dielectric constant has been changed from 78.5 to 17.7. It has been pointed out by Pitzer (29) that the value of ΔC_p ° for most of the electrolytes given in Table VI is of the order -40 calories. He also estimates that the entropy of ionization (first hydrogen only) of an acid should be of the order -22 calories. In a general way this agrees with the data given in Table VI.

10. Hemoglobin. Pauling (30) has explained the sigmoid oxygen saturation curve of ferrohemoglobin on the basis that interactions occur between the heme groups in such a manner that the free energy of oxygenation of one heme group is decreased by $RT \ln \alpha$ when an adjacent heme group is already combined with an oxygen molecule. α is termed the interaction constant. Its numerical value probably varies with the nature of the globin. It was concluded, since the oxygen equilibrium constant depends on acidity, that acid groups whose ionization constants are increased by oxygenation are coupled with the heme groups. The total heme-heme interaction energy in the molecule is about 6000 cal. per mole and the total heme-acid group interaction energy is about 6600 cal. per mole. On the assumption that the hemes are arranged at the corners of a square, the heme-heme interaction energy corresponds to the value 12 for the factor α . This represents the increase in the equilibrium constant of the heme-oxygen reaction when an adjacent heme is already in combination.

From a study of the equilibrium data for the reactions between ferrihemoglobin and hydrosulfide and azide ions, Coryell (31) has concluded that stabilizing heme-heme interactions occur in these systems analogous to those occurring in the ferrohemoglobin system with oxygen. The values for the total effective interaction energies, in calories per mole, are 3360, 3120, 0, and 0, respectively, for the equilibria with hydrosulfide, azide, fluoride, and hydroxide ions. In the first two equilibria, complexes with covalent ferric atoms are formed; in the last two, complexes with predominantly ionic ferric atoms are formed.

The ferro-ferrihemoglobin half-cell may be treated as an equilibrium between these two substances and electrons. This leads to the conclusion that the n in the Nernst equation when applied to these potential measurements is identical with the sigmoid coefficient, n, used in Hill's equation (32)

$$y = \frac{K'c^n}{1 + K'c^n} \tag{34}$$

for the sigmoid saturation curves of hemoglobin. In the present use of this equation K' and n are empirical constants, y is the fraction saturation of hemoglobin, and c is the concentration of a substance forming a compound with heme interactions stabilizing the molecule RT ln α cal. per mole of adjacent heme pairs. The fact that the value of n is in the neighborhood of 1.6 indicates that interactions occur in this system of hemoglobin compounds, both of which contain ionically bound iron. An interaction energy of about 2600 cal. per mole is involved.

A study of the oxidation-reduction potentials of the methemoglobin-hemoglobin system over the pH range 5-9 by Taylor and Hastings (33) shows that, by using a small amount of an electromotively active dye system as mediator (m-toluylenediamine indophenol, 1-naphthol, 2-sulfonate indophenol, o-chlorocresol indophenol, toluylene blue, or methylene blue), accurate values may be obtained. The curve obtained by plotting the values for E_h against per cent reduction at constant pH is not symmetrical, indicating that apparently the number of electrons involved in the reaction

vary from 1 to 2 as the oxidation progresses. No adequate explanation for this is, as yet, evident.

A plot of $E_{m'}$, the oxidation-reduction potentials of equimolar mixtures of hemoglobin and methemoglobin,

¹ In the equation as used by Hill, viz., $y/100 = (Kx^n/1 + Kx^n)$, y = percentage saturation of hemoglobin with oxygen, x = oxygen pressure, K = the equilibrium constant of the reaction, and n, the average number of molecules of hemoglobin in each aggregate, is a whole number >1.

$$E'_m = E_m + 0.0601 \log \frac{(H^+)}{K + (H^+)}$$
 (35)

where $E_m = +0.168$ volt, $K = 2.24 \times 10^{-7}$, and pK = 6.65 against pH shows that, between pH 5 and 6, the values of E'_m are independent of the pH. Between pH 6 and 7.5, the slope of the line undergoes a progressive change while between pH 7.5 and 9, the points lie on a straight line that has a slope of -0.06 volt per pH unit. The variation of the mid-point potential with pH indicates the presence in methemoglobin of a group whose apparent pK is 6.65. At pH 7 and 30°, the potential of an equimolar mixture of horse methemoglobin and hemoglobin is +0.139 volt.

Wyman (34) has made a study of the apparent heat of dissociation of oxyhemoglobin as a function of pH. The necessary data were obtained by titrating oxyhemoglobin with acid or base at several temperatures. When the apparent heat of dissociation is plotted against pH, the curve shows three plateaus which are separated by two transition regions whose mid-points lie close to pH 5.5 and 8.5 respectively. The dissociating groups of oxyhemoglobin fall into three classes on the basis of the heat of dissociation. Members of Class I which are carboxyl groups have an average apparent heat of dissociation of -2000 or -3000 cal. and are active in the acid range of titration. Members of Class II are active in the middle range and have an average apparent heat of dissociation of about +6200 cal. They represent imidazole groups. Members of Class III are active in the alkaline titration range, have an average apparent heat of dissociation of +11,500 cal., and represent either the amino groups of lysine or the guanidino groups of arginine.

The heat of the reaction of hemoglobin with oxygen has been determined by Roughton and his coworkers (35). The heat of combination of hemoglobin per gm. mole of oxygen or carbon monoxide was found to be independent of the percentage saturation within the limits investigated (25–80 per cent). The heat liberated per mole of oxygen at pH 6.8 is 9350 cal.; that at pH 9.5 about 13,000 cal. The difference was attributed to the heat of the dissociation due to formation of hydrogen ions which accompanies oxygenation at pH 6.8 but not at pH 9.5. Wyman (36) has studied the problem in order to determine how the heat of oxygenation varies over the range pH 3 to 11. He found that

$$\frac{Q_x - Q_0}{\Delta B_x} = 6500 \pm 650 \text{ cal.}$$
 (36)

Where Q_0 is the heat of oxygenation at some strongly acid pH (pH=0) where the acid dissociation is not affected by oxygenation, Q_x that at some other pH, and B_x is the number of equivalents of base bound at any given temperature and pH per mole of hemoglobin. The fact that $Q_x - Q_0$ is proportional to ΔB_x indicates that the variation in the heat of oxygenation with pH can be accounted for on the basis of the heat of the dissociation of hydrogen ion with which oxygenation is coupled. The 6500 value is the heat of dissociation of the base-binding groups that interact with the oxygencombining centers of the protein molecule. For ox hemoglobin $Q_0 = -11,700$ cal. Roughton's experimental value at 19° is 12,400 cal. with a possible error of about 1000 cal. Wyman's value for the heat absorbed due to the reaction of one mole of oxygen with one mole of horse hemoglobin at 20° and pH about 6.9 is 10,300 ±1000 cal. This value compares favorably with 9350 cal. at pH 6.8 found by Roughton. At pH 6.9 the change in the heat corresponding to a change in pH of 0.1 unit is about 550 cal. Of the 9350 cal. about 3000 cal. are due to the heat of solution of oxygen at 25°.

The free energy of the reaction does not depend on whether the oxygen is in solution or in the gaseous phase at the corresponding partial pressure. The change in free energy due to the combination of one mole of hemoglobin with 4 moles of oxygen, when the reactants are in the standard states, is given by

$$\Delta F = -RT \ln L_4^4 \tag{37}$$

where L_4 denotes the equilibrium constant for Hb(O₂)₄. If ΔF at any one pH is known, it is possible to calculate values for it over the whole pH range. At pH 8.3 and with a partial pressure of 1 mm. of oxygen, $\Delta F = +2200$ cal. For any other pressure, p mm., ΔF will be (2200–5450 log p) cal. The change with pH in the free energy of each stage of the oxygenation process is the same and equal to $1/4\partial\Delta F/\partial pH$. However, the free energy itself is different for each stage of the process due to interaction of the hemes as indicated by Pauling (30).

11. Carbonic and Carbamic Acid. Roughton (37) determined the increase of temperature with time (seconds) when a solution of hydrochloric acid was mixed with one containing sodium bicarbonate. From these data he calculated that, at 0° , ΔH_1 for $H^+ + HCO_3^- \rightarrow H_2CO_3$ is 1605 cal. and at 36.8° it is 685 cal. The value for ΔH_2 for $H_2CO_3 \rightarrow CO_2 + H_2O$ at 0° is 2880 cal. and at 36.8° it

Table VII
Solubilities of Certain Amino Acids in Water-Ethyl Alcohol Mixtures at 25° and the
Dielectric Constants of the Solvent and the Solution

Amino Acid	Eth Weight	anol Volume at 20°	Temp.	Density of Sat. Sol.	Solute per 100 gm. Solvent	N (mole fraction solute)		Constant (Solution)
	per cent	per cent	°C		gm.			
dl-Alanine	20.32	24.93	24.97	0.984	7.09	1.61×10^{-2}	66.70	83.29
	42.52	50.10	24.97	0.929	2.52	0.681×10^{-2}	53.45	59.26
	66.94	74.20	24.97	0.868	0.573	0.195×10^{-2}	39.60	40.86
	92.61	95.14	25.09	0.807	0.0329	0.152×10^{-8}	26.90	26.97
dl-Aspartic acid	20.00	24.55	25.06	0.963	0.266	0.410×10 ⁻³	66.99	67.42
	42.66	50.25	25.06	0.926	0.0992	1.81 ×10-4	53.40	53.56
	67.03	74.28	25.14	0.872	0.0317	0.724×10^{-4}	39.58	39.63
	92.61	95.14	25.07	0.807	0.0020	0.62 ×10 ⁻⁵	26.90	26.90
d-Glutamic acid	20.00	24.55	25.05	0.964	0.292	4.08 ×10 ⁻⁴	66.99	67.42
	42.66	50.25	25.08	0.925	0.131	2.17×10^{-4}	53.40	53.59
	67.11	74.35	25.07	0.867	0.0370	7.68 ×10 ⁻⁵	39.55	39.60
	92.61	95.14	25.04	0.807	0.0044	1.2 ×10 ⁻⁵	26.90	26.91
	100.00	100.00	25.04	0.783	0.0025	7.8 ×10 ⁻⁶	24.25	24.25
Glycine	20.32	24.93	24.97	0.994	8.72	0.232×10 ⁻¹	66.70	90.76
	42.52	50.10	24.97	0.931	2.47	0.793×10-2	53.45	60.24
	66.94	74.20	24.97	0.869	0.448	1.81 ×10 ⁻³	39.60	40.77
	92.61	95.14	25.09	0.806	0.0172	0.945×10^{-4}	26.90	26.94
dl-Leucine	20.32	24.93	24.97	0.964	0.493	0.772×10 ⁻³	66.70	67.52
	42.52	50.10	24.97	0.924	0.318	0.588×10^{-3}	53.45	53.95
	66.94	74.20	24.97	0.868	0.175	0.404×10^{-3}	39.60	39.86
	92.61	95.14	25.09	0.806	0.0258	0.808×10^{-4}	26.90	26.94
dl-Norleucine	20.32	24.93	25.69	0.965	0.625	0.977×10 ⁻³	66.70	67.74
	42.52	50.10	25.69	0.924	0.453	0.838×10 ⁻³	53.45	54.17
	66.94	74.20	24.97	0.868	0.266	0.613×10 ⁻³	39.60	40.00
	92.61	95.14	25.09	0.806	0.0417	0.131×10 ⁻³	26.90	26.96
dl-Serine	20.32	24.93	25.14	0.970	1.54	2.99 ×10 ⁻³	66.70	69.85
	42.52	50.10	25.14	0.923	0.461	1.06 ×10 ⁻³	53.45	54.36
	67.27	74.50	25.10	0.869	0.0840	2.43 ×10-4	39.35	39.51
	92.61	95.14	25.09	0.806	0.0028	1.1 ×10 ⁻⁵	26.90	26.90
dl-Valine	20.62	25.28	24.85	0.971	3.30	0.575×10 ⁻²	66.55	72.57
	43.36	50.99	24.85	0.924	1.53	0.317×10^{-2}	52.80	55.48
	67.11	74.35	24.93	0.870	0.570	0.147×10^{-2}	39.55	40.50
	92.61	95.14	25.04	0.807	0.0569	0.200×10^{-3}	26.90	26.99

(Dunn, M. S., and Ross, F. J., J. Biol. Chem., 125, 309 (1938).)

is 450 cal. The average energy of activation, k_0 , over the range 0-37° is 16,500 cal. The true first ionization constant, K_1 , of carbonic acid has a value 2.5 $(\pm 0.3) \times 10^{-4}$ at 0°. The velocity constant for the decomposition of free carbamic acid, determined by mixing ammonium carbamate with hydrochloric acid, was found to be about 80 at 0°. This corresponds to a half-life of about 0.009 sec. The ionization constant of the carboxyl group is 1.8×10^{-6} at

 0° . No indication was found either of combination of a second H⁺ ion with the $-\mathrm{NH_2}$ group in very acid solution or of zwitterion formation by the $\mathrm{NH_2}$ COOH molecule.

12. Solubilities of Amino Acids. Dunn and Ross (38) have determined the solubilities of certain amino acids in alcohol-water mixtures at 0° , 25° , 45° , and 65° . Only those values obtained at 25° are reproduced in Table VII. When the values for log N (mole fraction of the solute) are plotted against the reciprocal of the absolute temperature, both when the amino acids are in aqueous solution and when they are dissolved in 25 per cent alcohol by volume, the curves are nearly straight lines. It may therefore, be concluded that the deviations from perfect solutions are not of large magnitude. Some of the curves for the amino acids that were dissolved in 95 per cent by volume of alcohol show some deviation from straight lines, but the magnitude is not very large.

When $\log N$ is plotted against volume per cent of alcohol at constant temperature, the curves deviate considerably from straight lines, particularly at high concentration of alcohol. In order to obtain straight lines, the activity coefficients would have to increase to very large values with increasing concentration of alcohol. The slopes of the curves become more steep at constant molal concentration of the solute as the proportion of alcohol in the solution increases. It might be expected that the activity and osmotic coefficients would increase to very high values even at the low molal concentrations of solute.

When the values for the dielectric constants are plotted against the solubility values (moles per liter of solution) at constant temperature or against the temperature at constant alcohol concentration, the curves are linear only over a limited range.

13. Solubility of Salts in Solutions of Amino Acids. Keefer, Reiber, and Bisson (39) determined the solubilities of barium iodate and of calcium iodate in solutions of glycine and of alanine containing potassium chloride. The effects of ionic strength and of dipolar ions on the solubility of a salt were found to be independent and additive. The solubility of barium iodate or calcium iodate in glycine or alanine solutions is given by

$$\frac{1}{Z_1 Z_2} \log \frac{s}{s_{\infty}} = 0.505 \frac{(78.54)^{3/2}}{(D_d)} \frac{\mu^{1/2}}{1 + A\mu^{1/2}} + 0.0625 \frac{R^2}{a} (R^{\pm})$$
 (38)

where D_d is the dielectric constant of the dipolar ion solution, A, R, and a are constants, Z_1 , and Z_2 are the valences of the ions, and

 (R^{\pm}) is the molarity of the dipolar ions. The solubilities of barium iodate and calcium iodate in glycine solutions obey Kirkwood's limiting law (40) which predicts that, in dilute solutions (0.15 M found in this instance), $\Delta(1/Z_1Z_2\log s)$ should be a linear function of the dipolar ion concentration. When the logarithm of the solubility of silver iodate and of lead iodate in glycine solutions and in alanine solutions is plotted against amino acid concentration, the curves are not straight lines (41). This indicates that complexes between the metallic ions and the amino acid ions are formed. The following dissociation constants were obtained: K (silver glycinate) = 5.28×10^{-5} ; K (silver alaninate) = 1.37×10^{-5} ; K (lead glycinate = 6.7×10^{-6} ; K (lead alaninate) = 3.0×10^{-6} .

14. Activity of Glycine in KCl Solution. Roberts and Kirkwood (42) carried out electromotive force measurements at 25° of concentration cells with transference of the type

$$Ag |AgCl| KCl (m_1) |KCl (m_1), glycine (m_2) |AgCl| Ag$$

The molalities of potassium chloride and glycine, m_0 and m_2 , were varied over a range of from 0.05 to 0.50. At low values of m_2 , the activity coefficient of glycine, γ_2 , is given by

$$\log \gamma_2 = -0.1789 m_1 - 0.06278 m_1^2 + 0.1635 m_1^{3/2}$$
(39)

From the limiting slope of $\log \gamma_2$, -0.1789, the dipole moment of the glycine dipolar ion was calculated to be 14.4 Debye units.

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CHAPTER XVI

DIPOLAR IONIC STRUCTURE AND SOLUBILITY OF AMINO ACIDS, PEPTIDES, AND PROTEINS*

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1. Spectroscopic Studies of Dipolar Ions. The replacement of some or all of the hydrogen atoms in an organic molecule by deuterium gives rise to shifts in vibrational Raman frequencies. These often reveal relations to structure not readily observed otherwise. Certain substances containing an -NH₃+ group, such as the ions of hydrazine and hydroxylamine, show a broad diffuse band in the Raman spectrum near 2980 cm⁻¹. It is probable that such a frequency is present in all molecules containing this group, but in most cases it is unobservable because it is covered up by a far more intense frequency arising from a -CH₂ or -CH₃ group. In the methyl ammonium ion and in glycine, however, it is readily possible to convert the $-NH_3^+$ group into a $-ND_3^+$ group by the exchange reaction in heavy water (177, 178). Under these circumstances a broad diffuse band near 2180 cm⁻¹ appears, which is definitely associated with the -ND₃+ group. On the other hand, isoelectric glycine (+D₃N·CH₂·COO-) in heavy water shows no indication of the intense lines near 2450 and 2530 cm⁻¹ which are found to be characteristic of the uncharged -ND₂ group (178). (The latter correspond to frequencies near 3320 and 3380 cm⁻¹, found in compounds containing an uncharged -NH₂ group.) Thus the evidence from the vibrations of the -NH₃+ or ND₃+ group indicates clearly that glycine in heavy water is present as a dipolar ion.

The ratio of corresponding frequencies for vibrations of the NH₃+ group to those of the ND₃+ group is always very nearly 1.36; the same ratio holds also for the vibrations arising from the−NH₂ and −ND₂ groups. Other types of vibration in which hydrogen atoms are not significantly involved—for instance, those arising

^{*} For additional information, consult Cohn, E. J., and Edsall, J. T., Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions, New York, 1943. This footnote takes the place of that given on page 871.

from the -COOH or $-\text{COO}^-$ group—are altered by only about 1 per cent by deuterium substitution. Thus it is possible, by studying the effects of ionization and of deuterium substitution, to determine the origin of different types of frequencies in amino acids and related molecules. The substitution of deuterium for hydrogen in the $-\text{CH}_2$ group of malonic acid and the malonate ion has permitted the identification of certain frequencies near 1400 cm⁻¹ as arising from the ionized carboxyl group. Similar frequencies could also be observed in isoelectric amino acids, furnishing further evidence for their dipolar ionic structure (177).

2. Dielectric Constants of Amino Acids and Proteins: Variation with Frequency. Following the work of Onsager (46), Kirkwood (179) has given a general theory of the dielectric constants of polar liquids, based on statistical mechanics. In polar liquids the dipole moment of an individual molecule (μ) is, in general, different from its moment in the gaseous states (μ_0) , because the attractions of neighboring polar molecules alter its configuration. Conversely, the molecule under consideration affects its neighbors; in general, their orientation with respect to its electric moment is not random, because electrostatic forces and steric factors hinder free rotation of the molecule. Kirkwood defines a quantity $\bar{\mu}$, which is the total electric moment of a system composed of a specified molecule plus its neighbors. Only the first two or three shells of neighboring molecules need be considered; more distant molecules may be regarded as distributed at random with respect to the molecule under consideration. In general $\bar{\mu}$ may be somewhat different from μ, both in magnitude and direction, although they may be nearly the same in many liquids. If the angle between the vectors μ and $\bar{\mu}$ is denoted by θ , then Kirkwood's analysis for highly polar liquids leads to the relation between the dielectric constant (D) and the molar polarization (P):

$$D - 1 = \frac{9P}{2V} = 6\pi \frac{N}{V} \left(\alpha_0 + \frac{\mu \bar{\mu} \cos \theta}{3KT} \right) \tag{1}$$

V is the molal volume of the liquid; the other symbols have the same meaning as in equation (15) page 881. Kirkwood has shown that the high dielectric constant of water is well accounted for by equation (1) on the basis of the known dipole moment of water, and the orientation and mutual interaction of water dipoles in the liquid state. The equation is readily generalized for systems of two or more components, and leads, in the case of the amino acids

and peptides with their very high electric moments, to a simple approximate relation between the dielectric increment δ , of the solute, and the quantity $\mu \bar{\mu} \cos \theta$, at 25°:

$$\mu\bar{\mu}\cos\theta = 10.9\delta\tag{2}$$

Thus for glycine ($\delta = 22.6$ from Table IV, page 884), $\mu\bar{\mu}$ cos θ is 246, μ and $\bar{\mu}$ being expressed by Debye units. If we take μ as 15, from solubility studies (see page 921, where $\mu = 15$ corresponds to R = 3.15 Å), then $\bar{\mu}$ cos θ is 16.4, or only slightly greater than μ . If we define a mean moment μ_m by the relation $\mu_m = (\mu\bar{\mu} \cos \theta)^{1/2}$, then $\mu_m = 15.7$ for glycine. Similar estimates for other amino acids of higher dipole moment, and for peptides, give values of μ_m that are in satisfactory agreement with the values of μ calculated from the effect of salts on solubility.

Far-reaching advances have been made in the study of dielectric constant as a function of frequency. In a very slowly alternating field, dipole molecules follow the field, and remain in phase with it, except for the disorienting effects of Brownian motion. Thus, owing to the orientation of permanent dipoles, the dielectric constant is the same as in a static field. At extremely high frequencies, such as those of visible light, the alternation of the field is so rapid that the dipole molecules cannot follow it at all, owing to their high inertia; only the electrons in the molecules are so light that they remain in phase with the applied field, and the dielectric constant is given by the square of the refractive index. In some intermediate range, the dielectric constant, D, is found to vary as a function of frequency (ν) , according to the equation

$$D = D_0 - (D_0 - D_{\infty}) \nu^2 / (\nu^2 + \nu_c^2)$$
(3)

Here D_0 is the dielectric constant of the solution in a static field, D_{∞} is the dielectric constant at a frequency so high that the permanent dipoles of the solute cannot follow the field at all,³ and ν_c is determined by the size and shape of the molecule, the temperature, and the viscosity of the solvent medium. ν_c for most proteins in water is found to lie in the radio frequency range, very

¹ One Debye unit = 10^{-18} e.s.u.

² See also the recent and very thorough discussion of Conner, Clarke, and Smyth (179a), who have contributed also valuable new data on dielectric increments of amino acids and peptides.

³ It is assumed, however, that D_{∞} is measured at a frequency which is sufficiently low so that the solvent has the same dielectric constant as in a static field; that is, ν_c (solute) $\ll \nu_c$ (solvent), and D_{∞} is measured at a frequency between these two ν_c values.

commonly between 0.1 and 10 megacycles; for amino acids ν_c is much higher, generally above 1000 megacycles.

 $D_0 - D_\infty$, divided by the concentration of solute in gm./liter, is the dielectric increment of the solute per gram, E_t ; this, multiplied by the molecular weight, gives the molal dielectric increment, δ , already employed in Tables III and IV, page 882 and 884. The relaxation time, τ , of the solute is related to ν_c by the equation $\tau = (2\pi\nu_c)^{-1}$. Relaxation times of a number of amino acids and peptides are given in Table I; and dielectric increments and relaxation times of proteins in Table II. The relaxation time of a molecule is inversely proportional to the intensity of its rotary Brownian movement; the relaxation time increases with increasing size and increasing asymmetry of the molecule.

A large spherical molecule of radius r, in a medium of viscosity η , at temperature T, gives a single relaxation time:

$$\tau = \frac{4\pi\eta r^3}{KT} \tag{4}$$

so that knowledge of the relaxation time of such a molecule permits the calculation of the molecular volume $4\pi r^3/3$. An ellipsoid

Table I Relaxation Times, au, of Amino Acids and Peptides

Substance	$ au ext{obs.} imes 10^{11}$	$ au_0(ext{sphere}) imes 10^{11}$	
Glycine	2.6	6.2	
α-Alanine	6.6	7.9	
6-Alanine	6.7	7.9	
Diglycine	14.9	10.1	
Glycylalanine	20.3	11.8	
Alanylglycine	21.3	11.8	
Friglycine	13.0	14.0	
Lysylglutamic Acid	48.0	22.8	
Friglycine	18.1	14.0	
Tetraglycine	24.7	17.0	
Pentaglycine	36.3	21.9	
Leucylalanine	24.6	18.8	
Alanylglycylglycine	20.6	15.8	
Leucylglycylglycine	28.2	21.1	
Alanylleucylglycine	27.8	22.8	

The first six values are from Bateman and Potapenko (180) temp. 23.3°; the next two are from Wyman and Marcy (181) temp. 25°; the last seven are from Conner and Smyth (181a). For other relevant data on amino acids, see Fricke and Parts (182).

The calculated τ_0 values are derived from equation 4, calculating r from the apparent molal volume and electrostrictions given in Table VI, page 891 (Calculated by Dr. J. L. Oncley.)

	TABLE II	
Dielectric Increments, Dip	le Moments, Relaxation Times, and Calculated Axial Ratios	
	of Certain Proteins at 25°	

Protein	E_t	Mol. Wt.	μ	$ au_{ m H_2O} imes 10^8$	$\tau_0 \times 10^8$	a/b
Egg Albumin (183) (184)	0.17	44,000	250	18; 4.7	3.7	5
Horse Serum Albumin (185) (carbohydrate free)	0.24	70,000	380	36; 7.5	6.0	6
Horse CO-Hemoglobin (186) (184)	0.42	67,000	480	8.4	(6.6)	(1.6)
Pig CO-Hemoglobin (187)	0.3	(67,000)	(410)	13		
Horse Serum Pseudoglobulin $\gamma(188)$	1.14	142,000	1100	250; 28	22	9
Insulin (189)	0.38	40,000	360	1.6		
Lactoglobulin (190) (184)	1.58	40,000	730	15; 5.1	4.3	4
Edestin (188)	0.8	310,000	1400	240; 27	21	9
Gliadin (191)	0.10	42,000	190	27; 3.8	3.1	8
Secalin (192)	1.0	24,000	440	29; 2.7	2.1	10
Zein (193)	0.45	40,000	380	24; 4.2	3.3	7

 E_t =dielectric increment per gm. protein per liter; μ =dipole moment in Debye units (1 Debye unit = 10^{-18} e.s.u.); τ_{H_2O} is the relaxation time in water at 25° (correcting for the relative viscosity of water and the solvent actually employed); τ_0 =relaxation time of a sphere, of volume equal to that of the protein, in water at 25°; a/b=ratio of major to minor axis, calculated from τ_0 and observed relaxation times, by the equations of Perrin (194), neglecting hydration.

Insulin was studied in 80 per cent propylene glycol; lactoglobulin in M/2 and M/4 glycine in water; edestin in 2 M glycine in water; gliadin in 56 per cent aqueous ethanol; secalin in 54 per cent ethanol; zein in 72 per cent ethanol; other proteins in water. (This table is adapted from Oncley (193a)).

of revolution has two relaxation times, corresponding to rotation about the major and the minor axis (194). Correspondingly the equation (3) for D as a function of ν involves two values of ν_c , to each of which there is a corresponding dielectric increment; and the ratio of the two ν_c 's gives a measure of the deviation of the molecule from the spherical shape. For reviews, see Oncley, Ferry, and Shack (183) and Oncley (188). The method of dielectric absorption, which gives results equivalent to those of dielectric constant measurements by an entirely different technique, has been described and applied by Shack (184).

The dielectric increments of cystinyl-diglycine, cystinyl-di-diglycine, and diglycyl-cystine have been studied (195). The estimated dipole moments appear to be compatible with the assumption of free rotation around valence bonds in these molecules. A general method for calculating distances between specified groups in complex molecules capable of free rotation around valence bonds has been given by Wyman (196).

- 3. Heat Capacity and Electrostriction of Amino Acid Solutions. The charged groups of ions or dipolar ions, owing to electrostriction, "freeze" some of the water molecules in their neighborhood, and this leads to a decrease in heat capacity, similar to that which occurs when water is frozen to ice. Following the work of Zittle and Schmidt (74), Gucker, Ford, and Moser (197) made extremely accurate measurements of apparent molal heat capacity, $\Phi(C_p)$, in glycine and its uncharged isomer glycolamide at 5°, 25°, and 40°. At all temperatures, $\Phi(C_p)$ for glycine is lower than for glycolamide by 26-30 cal./deg./mole. A similar difference exists between α alanine and lactamide (Gucker and Allen (198)), while $\Phi(C_p)$ for β-alanine, owing to the greater separation of the charged groups, is about 20 cal./deg./mole lower than for α -alanine. These effects are of the order of magnitude that had been calculated earlier from other data (199). Further discussion is given in the Addendum to Chapter XV.
- 4. Solubility and Activity Cofficients of Amino Acids and Peptides. Kirkwood (200) has given the general theory of the activity coefficients of dipolar ion models, either spherical or ellipsoidal in shape, as a function of the ionic strength and the dielectric constant of the medium.

For a spherical dipolar ion of radius b, with the center of the dipole of moment μ located at the center of the sphere, he finds for the salting in coefficient (see equation (30), page 920) at low ionic strength:

$$\left(\frac{-\partial \log \gamma}{\partial \Gamma/2}\right) = \frac{2\pi N e^2}{2303DKT} \left(\frac{3\mu^2}{2DaKT} - \frac{b^3 \alpha(\rho)}{a}\right) \tag{5}$$

For the significance of the terms a and b, see Fig. 12 and equation (30), page 919.

The first term in the parenthesis represents a salting in effect, and in water at 25° becomes identical with equation (31) page 920. The second term represents a salting out effect, not derived in Kirkwood's earlier treatment; like the salting in effect it is electrostatic in origin. The function $\alpha(\rho)$ depends on the ratio $\rho = b/a$, as follows:

Table	III
$\rho = b/a$	$\alpha(\rho)$
0.0	1.00
0.2	1.01
0.4	1.08
0.6	1.21
0.8	1.54
0.9	1.96

The salting out term in equation (5) is calculated to be 0.08 for glycine and NaCl in water; a value that appears very reasonable in the light of the data given in Table XV, page 924.

Kirkwood also treats a dipolar ion model consisting of a prolate ellipsoid with charges +e and -e located at the foci. The salting in coefficient is found to be, for an ellipsoid of eccentricity $\epsilon = 1/\lambda_0$:

$$\left(\frac{-\partial \log \gamma}{\partial \Gamma/2}\right) = \frac{2\pi N e^4 g(\lambda_0) R}{2303 D^2 K^2 T^2} \tag{6}$$

The function $g(\lambda_0)$ is practically unity for an ellipsoid of eccentricity greater than 0.7; if the eccentricity is 0.33, g is 0.49. R is the dipole distance; that is, the interfocal distance.

This calculation neglects the salting out effect, and the finite size of the ions of the salt. However, it gives very reasonable values for R in some elongated amino acids and peptides of high dipole moment.

Kirkwood also considers a second ellipsoidal model in which a point dipole is located at one focus. This model is a useful approximation for α -amino acids with long side chains, such as valine and leucine.

Dunn and Ross (201) have reported precise measurements of solubility on eight amino acids, each studied in four different alcohol-water mixtures at four temperatures (see Chapter XV, Addendum). Keefer, Reiber, and Bisson (202) studied the solubility of barium and calcium iodates in glycine and alanine solutions, and calculated from Kirkwood's theory, dipole distances, R (see Fig. 12, page 919) of 4.07 Å for glycine and 3.74 Å for alanine. Silver and lead iodates were found to give abnormally high solubilities in glycine and alanine (203), and this was explained as due to complex formation between the metal ion and the glycinate or alaninate ion. Roberts and Kirkwood (204) have determined the activity of glycine in aqueous KCl solution, from E.M.F. measurements on concentration cells with transference, and accurate transport numbers; from these data, by Kirkwood's theory, they calculate the dipole moment of glycine as 14.4×10⁻¹⁸ e.s.u. The studies of Cohn, McMeekin, and Blanchard on the solubility of cystine, shown in part in Figs. 14, 15, and 16a of this chapter (see pages 922, 923, and 933), have been published in full (205) (206).

Cohn, McMeekin, Ferry, and Blanchard (207) have investigated the interactions of dipolar ions in aqueous solution, including glycine, diglycine, cystine, asparagine, and several others. They show that the activity coefficient γ_i of a dipolar ion, may be de-

Table IV
Solubility of l-Asparagine in Aqueous Amino Acid Solutions at 25°

Amino acid	Density Dielectri			ility of aragine	$\log N/N_0$	Log N/No
$egin{array}{cccc} { m concen-} & { m solution} \ { m tration} \ {\it C}_2 & ho \ \end{array}$		$egin{array}{c} ext{solvent} \ D \end{array}$	C_3	N	served)	lated)
		<i>l</i> -Aspa	ragine in	water		
moles per			moles per	mole		
liter 0.0	1.00714	78.5	liter 0.184	fraction 0.00336		
	<i>l</i> -Asparagi	ne in glycine.	$\delta = 22.6; K_1$	$R^* = 0.094; I$	$X_S^* = -0.00$)6
0.25	1.01544	84.2	0.194	0.00357	0.026	0.023
0.50	1.02336	89.8	0.199	0.00370	0.042	0.044
1.00	1.03886	101.1	0.211	0.00397	0.073	0.079
1.50	1.05383	112.4	0.225	0.00430	0.107	0.108
2.00	1.06861	123.7	0.231	0.00449	0.126	0.132
2.80	1.09080	141.8	0.247	0.00494	0.167	0.163
l	-Asparagine	in diglycine. δ	$=70.6; K_R$	*=0.136; K	$\zeta_s^* = -0.02$	9
0.25	1.02164	96.2	0.196	0.00364	0.035	0.035
0.50	1.03508	113.8	0.201	0.00380	0.054	0.061
1.00	1.06120	149.1	0.218	0.00425	0.102	0.101
1.40	1.08170	177.4	0.224	0.00449	0.126	0.125
<i>l</i> -Asp	paragine in ly	sylglutamic a	cid. $\delta = 345$	$K_R^* = 0.15$	$0; K_{\mathcal{S}}^* = -0$	0.075
0.098	1.01762	112.4	0.186	0.00346	0.018	0.018
0.192	1.02780	144.8	0.189	0.00356	0.030	0.030
	l-Asparagine	e in alanine.	$\delta = 22.6; K$	$R^* = 0.094;$	$K_S^* = 0.033$	3
0.25	1.01436	84.2	0.188	0.00347	0.014	0.014
0.50	1.02132	89.8	0.191	0.00357	0.026	0.025
1.00	1.03490	101.1	0.192	0.00368	0.040	0.040
1.50	1.04788	112.4	0.192	0.00375	0.048	0.049
<i>l</i> -Asp	aragine in α -	aminobutyric	acid. $\delta = 22$.6; $K_R^* = 0$.	026; $K_S^* = 0$	0.012
0.25	1.01382	84.2	0.182	0.00338	0.003	0.003
0.50	1.02025	89.8	0.180	0.00339	0.004	0.005
1.00	1.03347	111.1	0.178	0.00346	0.013	0.008
1.50	1.04591	112.4	0.170	0.00342	0.008	0.009

(Cohn, E. J., McMeekin, T. L., Ferry, J. D., and Blanchard, M. H., J. Phys. Chem., 43, 169 (1939).)

scribed as a function of the concentration, C_K , of another dipolar ion, by the equation

$$\frac{-\log \gamma_i}{C_K} = K_R * \left(\frac{D_0}{D}\right) - K_S * \tag{7}$$

Here D_0 is the dielectric constant of the solvent without component K, D is the dielectric constant of the solvent containing C_K moles of K per liter, K_R^* is a "salting in" constant, and K_S^* a "salting out" constant, analogous to the similar constants that characterize the interactions of dipolar ions with ions. The application of equation (7) to the interaction of several dipolar ions with asparagine is given in Table IV.

It will be noted that K_R^* increases with increase in electric moment of the dipolar ion acting as solvent, while increasing the length of the non-polar side chain of the solvent diminishes K_R^* and tends to increase K_S^* . In the interactions of asparagine and cystine with other dipolar ions, "change in free energy with change in moment increases by slightly less than the product of the first power of the moments" (207, page 187).

Equation (7) is applicable when component K is the same as i; that is, when the activity coefficient of a dipolar ion is studied as a function of its concentration. Fig. 1 shows the activity coefficient of glycine, asparagine, cystine, and hemoglobin (see 176), in glycine solutions, as a function of the glycine concentration. The studies of Smith and Smith (208, 209) on activity coefficients of amino acids in aqueous solution, from isopiestic vapor pressure measurements, have given important information in this field (see Chapter XII of the Addendum).

5. Solubility of Proteins. The use of solubility as a criterion of protein purity has been greatly refined by Northrop, Kunitz, Herriott, and their collaborators. Different amounts of protein are equilibrated, each with the same amount of a given solvent, and the quantity of protein dissolved is plotted as ordinate against the total protein in the system as abscissa. Until saturation with one component is attained, the resulting curve is of course a straight line inclined at 45° to the abscissa. If a single protein component is present, there is a discontinuous change of slope when saturation is attained; the curve then becomes horizontal, and the value of the ordinate gives the solubility of the protein in the solvent employed. The same type of curve may be obtained if the protein is a mixture of two solid phases, in amounts proportional to their

solubilities; or if it is a solid solution of two or more proteins having exactly the same solubility.

If two or more solid phases, of different solubility, are present, the curve will show breaks at several different points, each corresponding to the concentration at which saturation with one of the components has been achieved. When saturation with all components is attained, the composition of the solution is invariant,

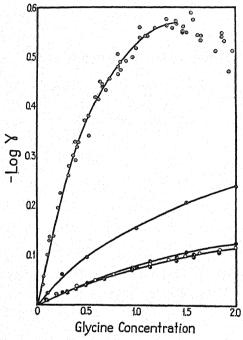


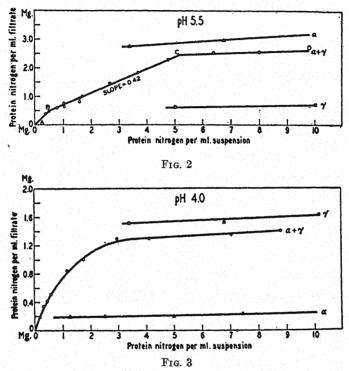
Fig. 1. Dipolar ions in glycine solutions. ○, glycine (Richards); ①, glycine (Scatchard and Prentiss); ③, glycine (Smith and Smith); ⑤, asparagine; ⊗, cystine; ⊙, hemoglobin (Richards).

(Cohn, E. J., McMeekin, T. L., Ferry, J. D., and Blanchard, M. H., J. Phys. Chem., 43, 169 (1939).)

and the curve is horizontal above this point. Between the initial section of unit slope, and the final section of zero slope, lie one or more segments of the curve with slopes between zero and unity. In employing solubility as a criterion of purity, it is especially important to study the portion of the curve that corresponds to the presence of only a very small amount of solid phase at equilibrium; for in the presence of a very large excess of saturating body the system may show a solubility independent of the amount of solid present, even if the solid is composed of several different phases.

These considerations are illustrated by Kunitz's solubility meas-

urements on α and γ chymotrypsin at pH 5.5, studied both separately and in combination (Fig. 2). Neither protein is quite pure, although both are nearly so; but the mixture of the two shows sharp breaks at B and C (Fig. 2, middle curve). If only the portion CD of this curve had been studied, it might have been taken as evidence for constant solubility by less careful and critical workers.



Figs. 2 and 3. Solubility curves of artificial mixtures of crystals of alpha and gamma chymotrypsin (40 per cent alpha+60 per cent gamma) in 0.4 saturated ammonium sulfate of pH 4.0 and 5.5 at 10° in the presence of increasing quantities of solid phase. At pH 4.0 the curve is of solid solution type while at pH 5.5 the curve corresponds to the theoretical curve of a mixture of two independent solid phases. Slope of BC: measured=0.42, calculated=0.43.

(Kunitz, M., J. of Gen. Physiol., 22, 226 (1938-39).)

In general, if the solid phase is a solid solution, the components do not dissolve in the same proportions in which they are present in the solid. Then the curve shows no sharp breaks, but passes fairly smoothly from the initial portion of unit slope to the final portion of zero or nearly zero slope. This situation is illustrated by Fig. 3, showing Kunitz's data for α -and γ -chymotrypsin, at pH 4.0. It is interesting that these two proteins form a solid solution at this pH, but are present as two distinct solid phases at pH 5.5

(Fig. 2). Solubility curves determined at several different values of pH, of salt concentration, or of temperature, and constancy of solubility as the protein is repeatedly re-extracted with the same solvent, serve to discriminate between the various possible types of systems. For the general principles involved, see (210), (211), and (212). Constancy of solubility in the presence of a large excess of saturating body is relatively insensitive as a test of purity; it is the form of the curve in the immediate neighborhood of discontinuities or bends that is most significant.

The first proteins to meet these criteria of purity were chymotrypsinogen (213) and trypsin (214). Some very recent measurements (211) on chymotrypsinogen fractions are shown in Fig. 4. After careful fractionation, a crystalline pepsin fraction was obtained, of constant activity and solubility (215). Similar studies, although somewhat less intensive, have been made on salmon pepsin (216), ribonuclease (217), and the luteinizing hormone (218). This work of Northrop and his school establishes solubility as probably the most delicate single test of protein purity now available; but it remains important to apply all available criteria of purity to protein preparations (cf. Pirie (219)), before considering them homogeneous in character.

The studies of Steinhardt on pepsin solubility (see footnote 32, page 947) have now been published in detail (220). Steinhardt expressed his results by the equation

$$S = A(1 - e^{-KV/A})$$
 (8)

where S is the total amount of protein dissolved by equilibration with the total volume V of solvent (whether the protein is treated with the solvent all at once or in many successive portions); A is the quantity of pepsin present at the beginning of the experiment; e is the base of natural logarithms, and K is a constant. Steinhardt found this equation to express his results very exactly for pepsin from two different sources, in a variety of solvents; and he indicated that it should be applicable to some of the data of Sörensen on other proteins. Under the conditions of Steinhardt's experiments, pepsin contains significant amounts of non-protein nitrogen, both in the crystals and in the solution in equilibrium with them. The more recent work of Herriott, Desreux, and Northrop (215) has shown that pepsin fractions of constant solubility may be obtained by working at pH values between 4 and 5, where the rate of formation of non protein products was much slower than in the more acid solvents employed by Steinhardt and other investi-

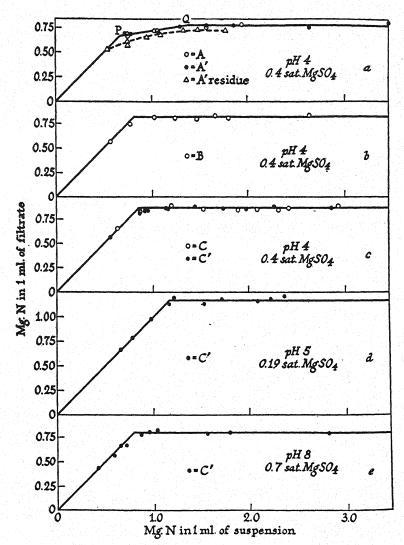


Fig. 4. Solubility curves of chymotrypsinogen fractions in various solvents. The fractions A and A', although nearly pure, give curves characteristic of solid solutions. The fractions B, C, C' give solubility curves of the type that would be expected for a pure single protein.

(Butler, J. A. V., J. Gen. Physiol., 24, 196 (1940).)

gators. McMeekin (221) obtained a crystalline carbohydrate-free serum albumin, which showed approximately constant solubility for considerable variations in the amount of saturating body.

Joseph (222) has developed a new type of amalgam electrode for determining the effect of proteins on the activity coefficients of salts. The cell employed is of the type

The amalgam of the metal Me is denoted by HgMe. The junction between the amalgam and the protein solution is made through a cellophane membrane, which is permeable to ions and water, but prevents reaction between the amalgam and the protein. If the salt $MeCl_z$ is present, at the same molality, m_3 , in the protein solution and the reference solution, then the E.M.F. of this cell is given by the equation

 $E_{p} - E_{0} = -\frac{\nu RT}{NF} \ln \frac{\gamma_{3}}{\gamma_{3}^{0}} \tag{9}$

Here ν is the number of ions formed by the dissociation of one molecule of salt, γ_3 is the mean ionic activity coefficient of salt in the protein solution and γ_3^0 is the same function for the reference solution. By measuring $E_p - E_0$ for various concentrations of salt and protein, the effect of the salt on the activity coefficient of the protein may be derived. Joseph thus studied the effect of calcium chloride on the activity of serum pseudoglobulin, gelatin, CO-hemoglobin, and serum albumin. In all cases the salt decreased the activity coefficient of the protein, the effect being greatest with pseudoglobulin and least with serum albumin. There is a general parallelism between the molal dielectric increments of these proteins and the effect of salt upon them. The observed effects agree well with those deduced from the effect of salts on the solubility of proteins, and are especially important because they provide an entirely independent method of determining the effect of salts on the activity coefficients of proteins.

The review by Cohn (223) of the properties and functions of the plasma proteins, contains much information relating to solubility studies.

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CHAPTER XVII

RELATION OF PROTEINS TO IMMUNITY

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Immunochemistry has maintained its rapid rate of progress since the first publication of this chapter and many of the most recent advances are concerned with proteins.

1. The Antigen. Although azo linkages permitted the combination of the most diverse substances with proteins and contributed greatly to the knowledge of protein specificity, more nearly "physiological" methods of attachment have been sought for and found (39, 40). In some instances injection of the modified proteins was followed by production of antibodies neutralizing the action of the reactive grouping (thyroxine or acetylsalicylic acid) introduced. The isocyanate method (41) has also been extended to the combination of carcinogenic hydrocarbons with proteins (42) and immunological as well as chemical and physical properties have been modified by introduction of phosphoryl groups (43).

Despite the importance of the specific polysaccharides in bacterial specificity, attempts to relate the often appreciable carbohydrate content of proteins to their specificity have failed. Possible reasons have been given (44).

With regard to the bacterial antigens progress has been made in the study of proteins of the hemolytic streptococcus (45, 46) and the tubercle bacillus (47).

A vast expansion has taken place in the knowledge of viruses, many or all of which are now considered to be proteins rather than living agents (48). Tobacco mosaic virus is a nucleoprotein of known dimensions and molecular weight, several other plant viruses have been crystallized and also characterized as nucleoproteins, and a number of animal viruses have been extensively purified and their particle sizes measured. A new tool, the electron microscope, has been of particular value in such studies and has yielded photo-

¹ The specific capsular substance of the anthrax-mesentericus group is a polypeptide of d(-)-glutamic acid (44a).

graphs showing forms which appear to be single virus molecules (see Fig. 3, page 1147). The dimensions found agree well with those obtained by x-ray, sedimentation, and other physical methods (49). Caution, however, in the interpretation of results with easily sedimentable material is indicated by the finding of similar large molecules in many normal tissues, where they may be carriers of phosphatase and the Wassermann and Forssman antigens (50). Electron micrographs showing antigen-antibody reactions have also been made (Fig. 1).

2. The Antibody (51). The protein nature of antibodies is now generally accepted since analytical (25, 17, 18, 24a, 51a), ultra-

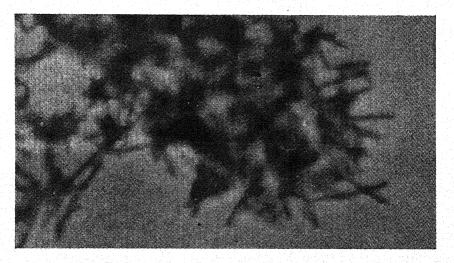


Fig. 1. Mixture of to bacco mosaic virus plus virus-antiserum several hours after mixing $(\times 48{,}000).$

(Anderson, T. F., and Stanley, W. M., J. Biol. Chem., 139, 339 (1941).)

centrifugal (19, 52), and electrophoretic (19, 53) measurements all show highly purified antibodies to be typical proteins. This provides a rational basis for the administration in diseases such as pneumonia and influenzal meningitis (54) of type-specific antibody in definite weight units, or milligrams of antibody nitrogen.

Antibodies have been found relatively resistant to the action of pepsin at pH>3 (55) but undergo partial degradation without loss of antibody function.

Evidence has been given that phagocytic cells of the liver and other tissues may give rise to normal serum globulin through the shedding of their surface films and that, in the presence of an antigen, characteristically modified globulins, namely antibodies, are produced (56). This would be compatible both with the earlier current theories (23) which did not fix the place or manner of antibody formation, and with a more recent graphic extension of the theory (57). In this, antibody function is considered due to the spatial configuration of the end groups induced by the presence of antigen, and antibody valence is, in general, limited to two. This view, if correct, also affords a basis for calculations and predictions concerning the precipitin reaction. Antibodies may also arise through modification by antigen of intracellular proteases providing the framework for partial replicas of themselves (globulins or antibodies) (51). This would permit antibody formation after destruction of antigen, progressive changes in the antibody with successive immunizations, and is not incompatible with (56).

- 3. Antigen-Antibody Interaction (51a). Earlier measurements of the velocity of antigen-antibody interaction involved delays and uncertainties owing to the time involved in centrifuging off the reactants. This difficulty was avoided by the device of competing reactions and it was found that the combination of egg albumin and horse anti-egg albumin to form a soluble compound was complete in less than 20 seconds at 0° (58). It has also been noted (59) that the heat of combination of hemocyanin with horse anti-hemocyanin, 40,000 calories per mole of antibody, or 3,300,000 per mole of hemocyanin (M.W., 7,000,000) is evolved to the extent of 80 per cent in 2 minutes. A three second reaction interval has been noted (58a).
- 4. The Precipitin Reaction. The equation for the typical precipitin reaction curve (page 966) may be derived with the aid of a different set of assumptions (60) than those originally used (25 g). In some instances, at least, the "curve" may be composed of a number of linear segments (61). Extended linear segments have also been noted in cross-reactions (62), again emphasizing the formation of antibodies of different reactivities as a result of injection of a single antigen.

Dissociation of specific precipitates for recovery of antibody has again been explored (63) and in some instances improved.

5. Complement Fixation and Immune Hemolysis. The entire aspect of the knowledge of complement or alexin and its function has changed as a result of two independent investigations. One, involving a thorough study of the behavior of the four components (C' 1, 2, 3, 4) of complement (C') has culminated in the isolation of the principal components in a state of high purity (64). The "mid-

piece" component, C'1, was found to be a euglobulin, precipitable at pH 6 and 1.39 M (NH₄)₂SO₄ concentration, with a sedimentation constant of 6.4×10^{-13} , and a mobility of -2.9×10^{-5} (PO₄^{\equiv}, $\mu = 0.2$, pH 7.7), $[\alpha]_D = -29^\circ$; carbohydrate content, 2.7 per cent. C'2, 4 were precipitated between 2.0 and 2.2 M (NH₄)₂SO₄ concentrations, could not be separated, and had the properties of a mucoeuglobulin; carbohydrate, 10.3 per cent, $[\alpha]_D = -193^\circ$, mobility, -4.2×10^{-5} . C'2 activity was destroyed in 30 min. at 50°, leaving the C'4 function unimpaired. In the other investigation (65) C'was measured in weight units by determination of the quantity of nitrogen added to certain specific precipitates from rabbit antisera and appropriate antigens in the presence of sufficient active C'. This has permitted establishment of weight and molecular relationships between hemolysin, C', and the red cell. It was also shown that in antigen-antibody combination as much C' as antibody, or even more, in weight units, could be taken up, and that this might be simply explained and fully accounted for in terms of the union of multivalent antigen with multivalent antibody (cf. pages 964–966, also (25)) providing for the first time a plausible hypothesis for the peculiar behavior of complement in being "fixed" by certain antigen-antibody reactions. Both investigations pointed to the same C' content for guinea-pig serum, namely, about 0.6 to 0.9 per cent of the total protein.

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CHAPTER XVIII

THE RÔLE OF PROTEINS IN NUTRITION

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1. Lysine. Confirmation has been obtained of earlier reports that lysine becomes a growth-limiting factor in heat-injured casein (53, 54). It has long been known that the same amounts of lysine can be recovered from both heated and unheated protein (55), proving that the lysine is not destroyed. Treatment with boiling alcohol also reduces the biological value of proteins, the *in vitro* digestibility, and the content of free amino groups, presumably the ϵ -amino groups of lysine (56).

The suggestion has been made that both heat and hot alcohol treatments of proteins, which are conditions favorable to dehydration, lead to the formation of new and enzymic-resistant linkages between free basic and amino groups in proteins (i.e. the ε-amino group of lysine and the free carboxy group of glutamic or aspartic acid). Thus the nature of the nutritive damage to lysine may be reconciled with the facts that, (a) analytically, lysine does not disappear and (b) both heat- and alcohol-treated proteins upon acid hydrolysis become equivalent in value to the hydrolyzed untreated proteins (57).

Aside from growth failure, little else has been observed as a consequence of lysine deficiency prior to the report that anemia in rats fed deaminized (nitrous acid-treated) casein may be rectified by the addition of an excess of lysine to the diet (58). In this case, the deficiency in lysine is due to the destructive action of nitrous acid on the ϵ -amino groups of lysine in proteins. The possibility remains that the anemia may have been caused by a harmful substance in deaminized casein which may be detoxified when an excess of lysine is added to the diet.

It is becoming increasingly evident that lysine occupies a unique position among the amino acids. For example, deuterium (H² or D) introduced as D₂O into the mouse was found in 9 of 10 amino acids isolated from mouse tissue proteins, the single exception being

lysine (59). Similarly, the introduction of ammonia or of certain amino acids containing N^{15} results in distribution of this isotope to many amino acids, except lysine. When given in the diet, lysine doubly marked by deuterium firmly attached to the carbon chain and by N^{15} in the α -amino group may be subsequently found combined in the tissue proteins, but with the *ratio* of both isotopic markers remaining unchanged from that of the original synthetic material. This indicates that no nitrogen from other sources is

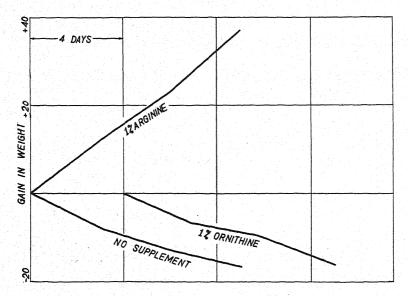


Fig. 1. Comparative growth curves of chicks, fed a diet containing arginine-free casein as the principal protein, showing the effects of arginine and ornithine supplements.

(Klose, A. A., Stokstad, E. L. R., and Almquist, H. J., J. Biol. Chem., 123, 691 (1938).)

transferred to lysine. Part of the synthetic lysine, however, is degraded since the isotopic nitrogen can be found in other amino acids of the tissue proteins (60). Lysine, once deaminated, evidently cannot be regenerated.

2. Arginine. The young rat is able to synthesize most of the arginine required for growth, while the adult rat can synthesize all of the arginine required for maintenance (61). Unlike the rat, the young chick lacks the ability to synthesize arginine, and rapidly loses weight on a diet very low in arginine (62) (see Fig. 1). Ornithine cannot replace arginine in the diet of the chick; however,

citrulline can do so effectively. It is evident that the chick can substitute an imino group for the oxygen of the ureido group of citrulline (63). In the chick, the Krebs-Henseleit cycle (Chapter V, page 221) for the regeneration of arginine from ornithine is not completed, failing in the step from ornithine to citrulline. The fowl, however, can produce ornithine, as shown by the fact that benzoic acid is conjugated with ornithine to form ornithuric acid (Chapter, V, page 221). This process probably takes place in the kidney, which contains some arginase, while the liver contains very little arginase. Creatine given in the diet of the chick will correct a partial arginine deficiency, presumably by an arginine-sparing action (64, 65) (Chapter V, page 221).

The damage to lysine and histidine in heated proteins poses a question of similar damage to arginine. Possibly, the ability of the rat to synthesize arginine has prevented disclosure of such damage, if any occurred.

3. Glycine. Although glycine, on the basis of conclusive evidence, has been classified as dispensable for the rat, a dietary complement of glycine is necessary for optimum growth of the chick (66, 67, 68). It is not known whether the chick is completely unable to form glycine from the normal components of the diet, or is merely unable to do so at a rate commensurate with the demands for early rapid growth. The fact that the fowl detoxifies benzoic acid by coupling it with ornithine rather than with glycine, as do other species, may be related to a difficulty in producing or mobilizing glycine.

The chick has the ability to synthesize glycine from acetates, as indicated by successful substitution of acetates for glycine in the diet (67). Creatine may also replace glycine (64, 65, 67) indicating that the glycine requirement, at least in part, is related to creatine formation in the chick (Chapter V, page 221). Terminal stages of glycine deficiency in the chick include low muscle creatine content, muscular underdevelopment, and profound weakness.

It is thus clearly established that, in respect to arginine and glycine, the requirements of the chick differ markedly from those of the rat. From such examples, the student of nutrition may be warned that reasoning entirely by precedent is decidedly unsafe.

4. Threonine. Of the four stereoisomers of α -amino- β -hydroxy-butyric acid only one, threonine, is capable of supporting growth of the rat (69).

5. Cystine¹, Homocystine, and Methionine. Nothing has appeared to shake the concept that methionine is strictly indispensable and can fulfill the functions of cystine, as well. The latter can exert noticeable biological effects only when the methionine intake is not too severely limited. There is no doubt, however, that cystine is physiologically indispensable and, in many cases, exerts a valuable sparing action on methionine.

Arachin from peanuts and, in fact, the total proteins of peanut meal are deficient in the sulphur-containing amino acids. Cystine does not significantly improve the growth-promoting properties of either arachin (70) or peanut meal (71). Methionine additions, however, lead to good growth.

In contrast to other proteins already mentioned, those of the soybean are improved by heating (72, 73, 74). Raw soybeans appear to contain a sulphur and nitrogen complex which is absorbable but can be used only poorly for tissue building. Heating the soybeans makes the complex more available (74). The deficiency of raw soybean protein is corrected to some extent by cystine, but most effectively by methionine (75). Heated soybean protein contains a positive, though suboptimal, quantity of available methionine (75).

Diets low in choline result in hemorrhagic lesions of the kidney in young white rats (76). These lesions are aggravated by cystine in the diet and delayed or prevented by methionine. In adult rats, such diets result in a fatty degeneration of the liver for which methionine, but not cystine, has a curative or lipotropic effect (77). Methionine must be present in a mixture of amino acids which will then maintain nitrogen equilibrium in the rat. Replacement of methionine with cystine in such a mixture leads to an increasingly negative nitrogen balance (61).

A level of 18 per cent casein in the diets of rats seems to have become so firmly fixed as to be almost traditional. There are a number of reports of improved growth of rats fed this level of casein plus added cystine (53, 77, 78), but in no case has it been shown that methionine would not improve growth as well. Such

¹ du Vigneaud, V., Brown, G. B., and Chandler, J. P., J. Biol. Chem., 143, 59 (1942) have shown that cystathionine may serve in lieu of cystine for the growth of rats. An enzyme is present in the liver capable of cleaving the thioether to yield cystine. Binkley, F., and du Vigneaud, V., J. Biol. Chem., 144, 507 (1942) found that the liver tissue of rats converts a mixture of homocysteine and serine to cysteine. According to Stetten, Jr., D., J. Biol. Chem., 144, 501 (1942) the carbon chain of serine is converted to cystine in the body of the rat.

results do show, however, that when casein is the sole source of protein for the rat, a protein level of 18 per cent is not optimum in at least one respect. It is not inappropriate to point out that the evidence for more than one "new" growth factor detected in crude preparations has been nothing more than the unwitting rectification of an amino acid deficiency.

The conclusive chapter in the cystine-methionine story is the strong evidence for the conversion of methionine into cystine. Rats grown on a cystine-deficient diet containing methionine produced and stored in the hair alone an amount of cystine vastly greater than that which could have been obtained as such from the diet (79). Radioactive sulphur (S³⁵) fed combined in methionine appears in tissue cystine but, when fed as sulphate, does not so appear. Cystine does not take up S³⁵ on exposure to hydrogen sulphide containing the isotope (80). This evidence amounts to proof of the direct conversion of the sulphur atom of methionine into that of cystine. The sulfur of methionine is used in part by the dog for the synthesis of taurine (94).

For the efficient replacement of methionine by homocystine, choline is required in the diet of the rat (81) and of the chick (70). The mechanism of this replacement undoubtedly involves a transfer of one of the labile methyl groups of choline to homocysteine, thus completing the synthesis of methionine. The dietary level of choline required for this process is several times that needed for growth (70). Choline may play an important part in the regeneration of methionine which has been demethylated through reaction with glycocyamine during the process of biological creatine formation (Chapter V, page 221). Ready in vitro transfer of methyl groups labelled with deuterium proceeds from choline to methionine, and vice versa (82).

- 6. Valine. Young rats fed diets in which the protein source consists of mixture of purified amino acids, except valine, experience sharp loss of appetite and weight and eventually die (83). Severe lack of muscular coordination and extreme sensitivity to touch are also apparent. This is one of the few examples of an amino acid deficiency syndrome which includes noticeably more than failure of appetite and growth. α -Hydroxyisovaleric acid may be substituted for valine in the diet (84).
- 7. Leucine, Isoleucine, and Tryptophane. The requirements of the rat for leucine and isoleucine may be met by the analogous α -hydroxy and α -keto acids (84). The l-tryptophane requirement for

optimum growth of the young chick is approximately 0.5 per cent of the diet (92).

8. The Fate of Dietary Amino Acids Investigated with Isotopes. Comprehensive reviews of the technique and the results of isotope studies with amino acids have been prepared (85) and detailed references on this subject need not be given. In particular, the classical studies of Schoenheimer and his collaborators have gone far toward clarifying and extending the understanding of amino acid and protein metabolism (see also Chapter V, Addendum).

Experiments with glycine, *l*-leucine, and *dl*-tyrosine marked with N¹⁵ and administered in the diet gave similar results in each case. Some of the isotope was found in the excreta, while half, or more, was introduced into the body proteins, but was distributed among many amino acids including glycine, tyrosine, leucine, glutamic acid, aspartic acid, proline, histidine, and arginine.

Leucine marked with N^{15} and deuterium, the latter firmly bound to the carbon chain, was found to have lost N^{15} and accepted N^{14} without relative loss of deuterium. The metabolism of leucine is evidently different from that of lysine. Histidine isolated from animals given isotopic ammonia was found to contain the isotope only in the α -amino group. Arginine contained the isotope principally in the guanidino group. In the case of most of the indispensable amino acids, deaminition and reamination may proceed in vivo, but not total synthesis.

Glutamic acid accepts nitrogen with extreme facility. Both deuterium and N¹⁵ are lost from labelled glutamic acid so rapidly when the labelled amino acid is fed to rats that little can be found in tissue glutamic acid. Glutamic acid rapidly takes up deuterium, under conditions of heavy and continuous dosage of the mouse with D₂O. The glutamic acid found in the tissue proteins of the experimental animals contained sufficient deuterium to indicate that most of the glutamic acid isolated had been newly formed during an interval of 24 hours. Phenylalanine is speedily converted into tyrosine by rats, as was found by tracing stably-bound deuterium in the former. The feeding of deutero ornithine was followed by the detection of relatively large amounts of deutero arginine and deutero proline in the proteins. These interconversions proceed rapidly despite steady and high intake of dietary glutamic acid, tyrosine, arginine, or proline, as the case may be, and are, hence, regarded as "automatic" reactions.

As consequences of the work with isotopes it becomes evident

that, (a) amino acids in the diet rapidly become incorporated in tissue protein, (b) coupled deamination and amination proceed readily in the case of most of the amino acids, (c) there is a continuous conversion of the carbon chain of some amino acids into others, (d) compounds newly formed by interconversion enter protein linkage in the same manner as those furnished by the diet, (e) these processes necessitate rapid opening and closing of peptide linkages in tissue proteins.

It seems probable, furthermore, that proteins may be interconverted without cleavage of peptide linkages through chemical changes involving the unbound groups of amino acids in the protein structure (86). Such possible changes include, for example, the loss of urea from the guanidino group of arginine; or the replacement of hydrogen by hydroxyl groups, as in the conversion of phenylalanine into tyrosine; or decarboxylation, as in the conversion of aspartic acid into alanine.

It has frequently been stated that the amino acids are the "building stones" from which proteins are made. This analogy leaves an impression of a static, permanent architecture which is not in accord with the newer information. Rather, the modern conception seems to be one of an extremely labile structure. The components of this structure are continually disappearing, reappearing, and changing into each other. The entire protein molecule, perhaps, may change into one of a different type, or function. The metabolism of the amino acids and the proteins probably cannot be described more specifically than as a "swirl of metabolism" or a "rapid metabolic merging of tissue and dietary components." The classical concept of a separate "exogenous" and "endogenous" nitrogen metabolism, for example, may require a critical revaluation, since it is not readily adapted to the newer knowledge.

9. Amino Acid Mixtures in the Nutrition of Higher Species. Studies with the dog have shown that amino acids dispensable for the growing rat are also dispensable for maintaining the adult dog in a state of nitrogen equilibrium (87). Dietary arginine is not required for the maintenance of the adult dog (87) nor of the adult rat (61). Evidently, both species synthesize arginine and otherwise exhibit similar amino acid requirements. These findings increase the probability that mammalian species other than the dog may have the same amino acid requirements as those of the rat.

In certain cases, when it is undesirable to feed native protein to infants, artificial digests of protein have been successfully used,

even by intravenous administration. A mixture of 20 amino acids has been employed in the nutrition of infants by the intravenous route. The nitrogen balances in the case of amino acid mixtures were as favorable as with the protein digests (88).

- 10. Avidin. The component of dietary raw egg white which produces a condition of severe dermatitis in experimental animals has been isolated in pure form as a protein (89). This protein has been called avidin because of its affinity for combining with and inactivating biotin, both in vitro and in vivo. The dermatitis symptoms observed in animals fed avidin (as raw egg white) are similar to those of known biotin deficiency and are curable by non-oral administration of biotin.¹ After coagulation by heating, the biotin-binding capacity of avidin is permanently lost (89). The "toxic" nature of raw egg white and the harmless nature of thoroughly cooked egg white are thus satisfactorily explained in terms of identified substances.
- 11. Specific Dynamic Action. The specific dynamic action of amino acids and proteins was first generally recognized as an apparently clear concept according to which an amino acid produced a definite heat increment in metabolism, varying from large to negligible among different amino acids. In time, this concept became obscure and obviously empirical. Eventually, the more fundamental features of the dynamic action have been uncovered. The metabolism of the carbon moiety of the amino acids is probably the major source of increased energy production following ingestion of amino acids or proteins (90, 91). In fact, the closest correlation found between any two measurable factors is that between the dynamic effects and the total metabolizable energy of the amino acids. These findings "do not lend support to the idea that certain amino acids or certain of their cleavage products act in the body as special metabolic stimulants in the pharmacodynamic sense" (91). Stripped of its mysticism, the specific dynamic action
- ¹ According to Hofman, K., Kilmer, G. W., Melville, D. B., and du Vigneaud, V., J. Biol. Chem., 145, 503 (1942), biotin has the structure:

of amino acids and proteins is found to be merely the heat evolved during the intermediary metabolism of these substances.

12. The Nutritional Importance of Amino Acids in the Complete Life Cycle. Of necessity, research work on the nutritional importance of the amino acids has been limited to small scale experiments because of the cost and scarcity of amino acids and the difficulty of preparing adequate diets in which all sources of protein could be considered well known. Furthermore, the majority of the work done has been conducted on the amino acid requirements of the young animal for the attainment of satisfactory rates of growth, since rates of growth are convenient and usually sharp criteria of nutritional completeness. It is obvious that the requirements of the young growing animal must be satisfied before much of significance can be done with adult animals.

A good start has been made on the amino acid needs for maintenance. It is apparent that certain qualitative differences exist between growth and maintenance needs. The adult rat, apparently, needs only threonine, isoleucine, tryptophane, valine, methionine, either phenylalanine or tyrosine, and either leucine or norleucine to prevent a negative nitrogen balance and to maintain body weight (61). Lysine, leucine, histidine, arginine, and phenylalanine, all required for optimum growth, need not be present in a maintenance diet, at least for short periods of time.

Information on the amino acid requirements for productive functions, such as milk secretion and egg laying, is extremely scanty. The relations of the amino acid composition of the diet to longevity (93) and to reproduction are additional problems worthy of much attention. These aspects of amino acid requirements are becoming increasingly feasible for study, in so far as other accessory food factors, especially the vitamins, are being recognized and conveniently provided for in pure or non-protein-bearing forms.

The fact that phosphoric acid and protein as well as certain of the vitamins are the components of coenzyme systems indicates that the first two play a rôle equally important to that of the vitamins. An adequate intake of all three is necessary for nutritional purposes.

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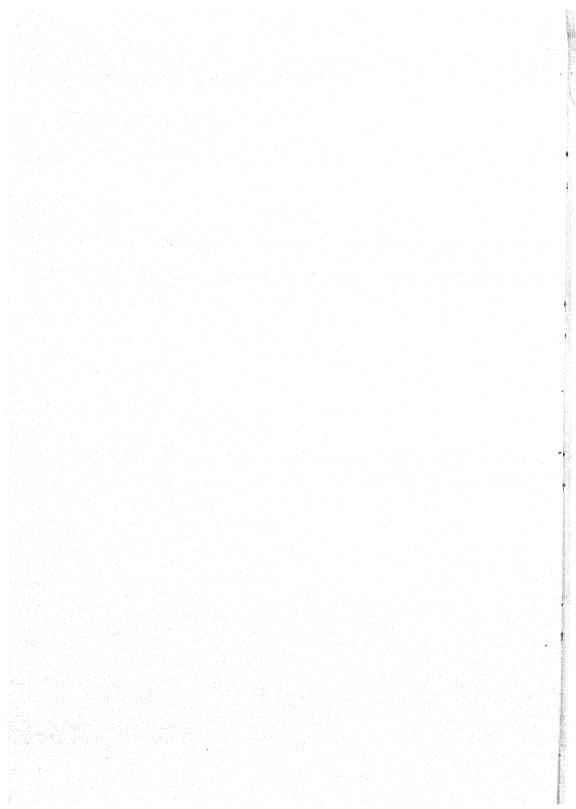
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Postscript. Since the above was written the following information has become available:

- 95. Rose, W. C., Haines, W. J., and Johnson, J. E., J. Biol. Chem., 146, 683 (1942) have reported that the amino acids, previously shown to be dispensable for rats and dogs, are also dispensable for man, while methionine and valine are indispensable.
- 96. Holt, Jr., L. E., Albanese, A. A., Brumback, Jr., J. E., Kajdi, C., and Wangerin, D. M., Proc. Soc. Exp. Biol. and Med., 48, 726 (1941) as well as Cox, Jr., W. M., Mueller, A. J., and Fickas, D., Proc. Soc. Exp. Biol. and Med., 51, 303 (1942), have reported that tryptophane is indispensable for the maintenance of nitrogen equilibrium in man. Albanese, A. A., Holt, Jr., L. E., Brumbach, Jr., J. E., Hayes, M., Kajdi, C., and Wangerin, D. M., Proc. Soc. Exp. Biol. and Med., 48, 728 (1941) have reported, on similar grounds, that lysine is indispensable in human diets.



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